Investigations on the biodegradation of polyesters by isolated mesophilic microbes

Untersuchungen zum mikrobiellen Abbau von Polyestern durch mesophile Mikroben

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El-Sayed Belal Abd El-Monteleb Belal
aus Kafr El-Sheikh, Ägypten

1.	Referent:	Prof. Dr. W.-D. Deckwer
2.	Referent:	Prof. Dr. H. H. Hanert

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Symbols and Abbreviations

A	area	[cm²]
APS	Ammonium per-sulfate	–
Asp	Aspartate	-
ASTM	American Society for Testing Materials	-
B	1,4-Butandiol	-
BSA	Bovine serum albumin	-
BTA	Copolyester consisting of 1,4-Butandiol, terephtalic acid, and Adipic acid	-
Ø	Diameter	[cm]
D	Day	-
Da	Dalton	-
DIN	Deutsches Institut für Normung	-
DNA	Desoxyribonucleic acid	-
rDNA	Ribosomal desoxyribonucleic acid	-
DSMZ	German Culture Collection	-
DTT	Dithiothreitol	-
g	Gram	[g]
FPLC	Fast Protein Liquid Chromatography	-
GBF	Nation Research Center of Biotechnology (Gesellschaft für Biotechnologische Forschung, GmbH)	-
G+C	Guanidine + Cytosine	-
Gly	Glycine	-
GPC	Gel permeation chromatography	-
h	Hour	-
His	Histidine	-
l	Liter	-
ISO	International Standerization Organization	-
min	Minute	-
Mol	Mol	-
MSV	Mineral Salt Vitamin medium	-
n	Number	-
PAGE	Poly-acrylamide Gel Electrophoresis	-
PCL	Poly(εcaprolactone)	-
PCR	Polymerase Chain Reaction	-
PHA	Polyhydroxyalkanoates	-

PHB	Poly(β-hydroxybutyrate)	-
PHBV	Poly(β-hydroxybutyrate-co-β-hydroxyvalerate)	-
PVC	Poly(vinylchloride)	-
RNA	Ribonucleic acid	-
rpm	Round per minute	-
SDS	Sodium-dodecyl sulfate	-
Ser	Serine	-
t	Time	-
T	Terephthalic acid	-
T_m	Melting temperature	[°C]
TEMED	N,N, N´N`-Tetramethylethylendiamin	-
TSB	Tryptic Soy Broth	-
UV	Ultra violet	-
V	Volume	[l]
X	Variable for any given amino-acid	-

1. Introduction and aim of the work

Synthetic polymers - designated as plastics - are applied in a wide range of packing-, household, agricultural, marine and architectural applications. Plastics were developed as light-weight, and durable materials, and they have replaced natural resources, such as metals and stones. However, its properties of durability have caused serious problems since plastic waste accumulates in the environment. The accumulation of abandoned plastics has caused a global environmental problem. Nature usually can not handle plastic waste, since the majority of plastics is not degraded by microorganisms. At present, about one hundred million tons per year of plastics are produced in the world. With the increase in production, the amount of plastics wastes has raised enormously (Mukai, et al., 1995) and increased the costs of solid waste disposal (owing to the reductions in available landfill space) dramatically. Alternatives to waste disposal such as plastic recycling are quite limited from an economical view point and partially include potential hazards (such as dioxine emission from PVC incineration).

Biodegradable plastics opened the way for new waste management strategies since these materials are designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities. Most of the plastics on the market, claimed to be biodegradable, are based on synthetic and microbial polyesters (Augusta et al., 1992; Witt et al., 1997). Polyesters are potentially biodegradable due to the hydrozable ester bonds. In addition, they combine several properties that make them attractive candidates for various industrial applications. Cellulose and starch as natural polymers are degraded simply by microorganisms under environmental conditions. Within the natural polymers is polyhydroxybutyrate (PHB), which are produced intracellularely by various microorganisms. The PHB was available on the market under trade name “Biopol”. The commercial polyesters product of Biopol was recently stopped, probably due to the high price level of this microbial polyester and the variation in PHB properties is limited. Beside the natural polyester PHB, a number of the synthetic aliphatic polyesters such as poly(ε-caprolactone) (PCL) are produced as commercial products. Aliphatic polyesters exhibit usually good biodegradability, but as e.g. PCL they exhibit a significant disadvantage- the low melting temperature of about 60°C- excluding it from many applications (Witt et al., 1997; Müller et al., 1998)

In contrast to most aliphatic polyesters, aromatic polyesters like poly(ethylene terephthalate) (PET) provide excellent material properties and, hence, are commercially widely used (e.g. bottles). However, up to now these polymers are considered as resistant against microbial

attack (Huang 1989, Aminbhavi et al., 1990, Kawai 1995). With the intention of combining both, biodegradability and good material properties, copolyester containing aliphatic as well as aromatic monomer were tested as biodegradable materials. The combinations of terephthalic acid (30 to 60 %), adipic acid and 1,4-butandiol ("BTA-polymers") turned out to be the most appropriate combination both, with regard to the material properties (flexible films, melting point from 90-140°C) (Witt et al 1995). In 1998, the BASF AG in Germany started to produce a BTA-based copolyester on a commercial scale, which is now on the market under the trade name Ecoflex®This product exhibits properties similar to polyethylene and the price is quite low for the raw material (3-4 €/kg). The Eastman company (USA) is announcing another BTA-modification; the product is called 'Eastar Bio'. These two aliphatic-aromatic copolyesters overcome the disadvantages of many aliphatic materials (Müller et al., 2001; Witt et al., 2001).

The intention was that these materials would reduce waste deposit volume while undergoing degradation in a landfill, or alternatively they could be treated in composting plants. In combination, these technologies offered a new approach to the management of plastic waste. The treatment of biodegradable plastics in composting processes and biowaste is discussed as an alternative to plastics recycling or incineration.

The biodegradability is evaluated with laboratory test methods monitoring the metabolism of the material by CO_2 or O_2 consumption. Two test methods are based on synthetic aqueous media (DIN V 54900 Part 2; 14851; ISO 14852), including the possibility of establishing a carbon balance and to regard the carbon used for the formation of new biomass as degraded. The preferred test method, however is the so-called controlled composting test (Gu et al., 1993; Pagga et al., 1995; Starnecker and Menner, 1996; Degli-Innocenti et al., 1998: Touminen et al., 2002), using mature compost at around 60°C as a matrix.

The relevance of aquatic tests in a test scheme focused on a compost environment is discussed by Van der Zee et al. (1998), because many polymers have been proved to degrade more slowly in aqueous media than in compost. However, the aim of this test is not to simulate composting conditions (the controlled composting test also uses mature compost instead of biowaste) but rather to prove in principle the biodegradation.

Problems of the controlled composting test include background CO_2 evolution, which can be affected by the presence of plastics (priming effect) and the missed opportunity to determine carbon balance with sufficient accuracy. Attempts to solve these problems include referral of the biodegradation calculated from CO_2 evolution to the CO_2 release of degradable references substance (e.g. cellulose) or to use an inert solid matrix inoculated with eluate from compost instead of mature compost (Bellina et al., 1999, 2000).

In the test schemes, limit values are also given for the evaluation (Pagga, 1999). The maximum test duration is 6 months (1 year for radiolabeled material in ASTM D6400). In real biowaste it is not possible to perform respirometry measurements with sufficient accuracy, and so in a real compost environment only disintegration is evaluated. There is no demand to complete the biodegradation (metabolism) of the plastics during the composting process, but the final degradation can take place in the soil where the compost is applied (during compost use). Disintegration testing can be performed in laboratory simulation test using controlled composting reactors of several hundred liters content (Itävaara et al., 1997) simulation tests or tests in a real composting plants (only mandatory for DIN V 54900). The test duration ranges from 5 weeks (ASTM, extendable) to 15 weeks (DIN). The degree of disintegration is analyzed by sieving the compost; in all standards a maximum fraction of 10% of polymer fragments larger than 2mm is demanded.

The general requirement that the quality of the final compost is not negatively affected is covered by the established national test methods ensuring compost quality. Criteria for this include compost maturity, visual impurities, density, pH, nutrient content, salt content, and heavy metal content. Additionally, ecotoxicity tests focused on plant growth are part of the compost quality characterization (e.g., plant tests according to OECD guidelines OECD 208). Additional toxicity investigations such as earthworm tests, tests with luminescent bacteria, tests with *Daphnia magna*, or fish tests were discussed during the development of the standards, but due to the limited experiences with these (Dang et al. 1996, Fritz, 1999; Degli-Innocenti et al., 2001: Witt et al., 2001; Tuominen et al., 2002) in combination with compost, such experiments were not generally included (ASTM includes earthworm testing according to OECD guideline OECD 207).

Following an initial focus on the biodegradation of plastics in landfills and composting processes, biodegradation in soil has received intense interest based on the use of biodegradable plastics in agriculture (e.g., as mulching films or as matrices for the controlled release of nutrients or pesticides). However, in contrast to the evaluation of compostability, the standardization of test methods to monitor the degradation of plastics in soil faces serious problems:

- While composting is a technical process where parameters as pH, temperature, humidity and biowaste composition are maintained within defined limits to guarantee an optimal process, the soil types and environmental conditions can vary widely significantly, and usually cannot be controlled in nature. Standards must take into account these variations in some way.
- Due to biological activity and higher temperatures, the biodegradation of plastics composting usually occurs more rapidly than in a natural soil environment. From a technical

viewpoint it will be more complicated to monitor the slow degradation of some plastics in soil, with sufficient accuracy.

Due to the variability in conditions and difference in applications, the test scheme will most likely be more complex than the standards for compostability. Determining the metabolism via CO_2 detection in soil can be problematic due to the usually slower degradation rates, combined with a certain background CO_2 production from the soil. A method for CO_2 detection in closed system with minimized amount of soil was proposed by Solaro et al. (1998); alternatively, ^{14}C-labeled samples can be used (Albertsson, 1978). In addition to possible low CO_2 evolution rates, the determination of a carbon balance in soil is problematic, and influences of the added polymer on background CO_2 evolution have been observed (Sharabi and von Bartha, 1993). An additional problem centers on the evolution of disintegration behavior under real life conditions. The influence of soil characteristics and environmental conditions on plastics has been examined (Calmon et al., 1999; Ho and Pometto, 1999, Nishide et al., 1999; Grime et al., 2000) and the question has arisen as to which type of soil should be used to characterize degradation behavior as representatively as possible.

In the biodegradation tests mentioned above, undefined mixed cultures were used. On the other hand, different previous studies were performed in our working group on the biodegradation of polyesters by individual strains under different conditions. Kleeberg et al., (1999) isolated thermophilic actinomycetes degrading BTA copolyester. The authors found that two actinomycetes strains exhibited about 20 fold higher BTA degradation rates than usually observed in a common compost test. Abou-Zeid et al., (2001) isolated different anaerobic bacterial degrading natural polyesters (PHB and PHBV) and synthetic aliphatic polyesters (PCL and SP 4/6) under anaerobic conditions.

Therefore, the aim of the present work is concentrated on the biodegradation of two groups of the synthetic polyesters under mesophilic conditions by isolation of individual strains which are able to degrade the two groups. The first group are aliphatic-aromatic copolyester such as BTA 45:55 (which is produced commercially under trade name Ecoflex in BASF, Ludwigshafen, Germany) and BTA 40:60, the second group are synthetic aliphatic polyesters respentative in Poly (ε-Caprolactone) (PCL).

In order to realize the aim mentioned above, this work focused on the following main tasks:

1) Isolation of microorganisms with efficient degradation activity for different polyesters out of the two groups: synthetic aliphatic polyesters (PCL) and synthetic BTA copolyesters. The microorganisms with the highest polyester degradation activity were isolated and selected for further investigations such as

● Determination of the polyester substrate specificity of the different microorganisms for SP 4/6 (aliphatic counter part for BTA-copolyester) and also PHBV as natural copolyester.

● Optimization of the cultivation conditions for the efficient degrading isolates.

● Characterization and identification of the most promising organisms.

2) Use of the isolates for degradation test under natural conditions.

3) Isolation and characterization of the microbial polyester degrading enzyme(s).

2. Background and Literature Review

2.1. Biodegradable polymers

The current worldwide demand for plastics is in excess of million tones per year (Rapra Technology Limited, 1992). The rapid growth in consumption of plastics in recent years has lead to concerns from consumers, environmentalists and indeed the plastic industry, regarding the effective management of post consumer waste and greater use of and dependence on fossil fuels. The emphasis now is on minimizing the unnecessary use of plastics and on developing methods for recovery and recycling plastics waste. Alongside and compatible with these, much work is carried out into different ways to reduce the environmental impact of plastics. One way of doing this could be the use of biodegradable plastics.

Generally, conventional technical and synthetic polymers such as polyethylene and polystyrene are not biodegradable (Aminabhavi et al., 1990). Natural biodegradable polymers like protein, cellulose, starch, lignin and even the natural polyester PHB, have a backbone of carbon atoms interrupted by hetero-atoms such as nitrogen and oxygen in the main polymer chain. These heteroatoms represent potential points of attack for enzymatic hydrolytic and oxidative cleavage (Timmins and Lenz, 1994). Generally, polymers which contain double bonds or ether, ester and peptide bonds in the backbone such as natural rubber, polyethers, polyester and polyamides, respectively, are more or less biodegradable. However, one exception of the rule is the example of poly(vinylalcohol) (PVOH) as a biodegradable polymer with pure C-C bonds. In this case, degradation proceeds via primary oxidation of the hydroxyl groups, followed by polymer chain cleavage similar to fatty acid degradation (Sakai et. al,1986).

In the 1970s, work was started in US and elsewhere to develop photo-degradable and biodegradable plastics for the packing industry. The requirements for such polymers were:

1) Non toxic materials with non toxic degradation products that would not affect the drainage water from landfills;
2) Polymers with suitable mechanical properties for specific uses;
3) Economic viability;
4) Degradation control of the plastics via polymer modification;
5) Process ability.

The source of biodegradable materials is mainly of two types: natural material known to biodegrade and special synthetic biodegradable polymers. The so called biodegradable polymers can, therefore, be classified into four groups after Witt et al., (1997) as follows:

1) natural polymers
2) chemically modified natural polymers
3) synthetic polymers composed from natural building blocks
4) synthetic polymers from petrochemical building blocks

Table (2.1.) lists the advantages and disadvantages of each group giving examples for each. Synthetic and microbial polyesters form the largest number of reported types of biodegradable polymers and are the main focus of the present study.

Table 2.1. Classification of the biologically degradable polymers based on the polymers source after Witt et al., (1997).

Source of Polymer	Advantages	Disadvantages	Examples
Natural polymers	Renewable resources, mostly low-priced	Low reproducibility and variability of material properties	Starch, cellulose, PHB
Modified natural Polymers	Partially from renewable resources, variability of Material properties	Expensive, structure control is difficult	Cellulose- acetate, starch acetate
Fermentatively Produced Monomers	Renewable resources, good reproducibility of material properties and structure control	the monomers are expensive such as 1,3-propandiol, or succinic acid	PLA
Petrochemically produced monomers	Good material properties, good reproducibility of material properties, inexpensive, fast synthesis	No renewable resources	Polyester amides polyester urethanes, aliphatic homopolyesters, aliphatic-aromatic copoylesters

Table (2.2.) shows commercial biodegradable polyester (Gross, R. A. and Kalra B. 2002).

Table 2.2. List of biodegradable polyester produced commercially

Material	company	Installed capacity (tons/year)
Poly(hydroxy butyrate-co-hydroxy valerate)	Metabolix	?
Poly(lactic acid)	Cargill Dow LLC	140,000
Poly(ε-caprolactone)	Union Carbide	>5.000
Poly(butanediyl succinate)s	Showa Denko	3.000
Poly(ester amide)	Bayer	?
Poly(ethylene terephthalate, adipate)	Eastman Chemical	15.000
Poly(ethylene terephthalate, adipate) (Ecoflex)	BASF	8.000

Current research interest in biodegradable polymers is connected with well-defined areas of use. Biodegradable plastics offer one solution of managing of the solid wastes and are used for various application Target markets for biodegradable polymers include packaging materials (trash bags, wrappings, loose-file foam, food containers, film wrapping, laminated paper), disposable nonevents (engineered fabrics),hygiene products (diaper back sheets, cotton swabs) and consumer goods (fast-food tableware, containers, egg cartons, razor handles, toys).

2.2. General mechanism of biodegradation and definitions

2.2.1. General mechanism of biodegradation under aerobic conditions

The biodegradation of polymers normally refers to an attack by microorganisms on non-water-soluble polymer based materials (plastics). This implies that the biodegradation of plastics is usually a heterogeneous process. Because of a lack of water solubility and the size of the polymer molecules, microorganisms are unable to transport the polymeric material directly into the cells where most biochemical processes take place. The microorganisms must first excrete extracellular enzymes which depolmerize the polymers outside the cells (Fig.2.1.). As a consequence, if the molar mass of the polymers can be sufficiently reduced to generate water–soluble intermediates, these can be transported into the microorganisms and fed into the appropriate metabolic pathway (s). As a result, the end product of these

metabolic processes include water and carbon dioxide together with new biomass. The extracellular enzymes are too large to penetrate deeply into the polymer material and so act only on the polymer surface. Consequently, the biodegradation of plastics is usually a surface erosion process.

Although the enzyme- catalyzed chain length reduction of polymers is in many cases the primary process of biodegradation, nonbiotic chemical and physical process can also act on the polymer, either in parallel or as a first stage solely on the polymer. These nonbiotic effects include chemical hydrolysis, thermal polymer degradation and oxidation or scission of the polymer chains by irradiation (photo degradation). For some materials, these effects are used directly to induce the biodegradation process [e.g. poly(lactic acid), pro-oxidant modified polyethylene], but they must also to be taken into account when biodegradation is caused predominantly by extracellular enzymes. Because of the coexistence of biotic and nonbiotic processes, the entire mechanism of polymer degradation could in many cases also be referred to as environmental degradation.

Environmental factors do not only influence the polymer to be degraded, they also have a crucial influence on the microbial population and on the activity of the different microorganisms themselves. Parameters such as humidity, temperature, pH, salinity, the presence or absence of oxygen and the supply of different nutrients have important effects on the microbial degradation of polymers and so these conditions must be considered when the biodegradability of plastic is tested.

Another complicating factor in plastics biodegradation is the complexity of the plastic materials with regard to their possible structures and compositions. In many cases plastics do not consist simply of only one chemical homogenous component, but contain different polymers (blends) or low molecular weight additives (e.g., politicizes). Moreover, within one polymer itself different structural elements can be present (copolymers), and these may either be distributed statistically along the polymer chains (random copolymers), distributed alternately (alternating copolyesters), or they may also build longer blocks of each structure (block-copolymer). Another structural characteristic of a polymer is the possible branching of chains or the formation of networks (cross-linked polymers). These different structures of a polymer, despite having the same overall composition, can directly influence accessibility of the material to the enzyme-catalyzed polymer chain cleavage and also have a crucial impact on higher-ordered structures of the polymers (crystals, cristallinity, glass transition) which have been shown predominantly to control the degradation behavior of many polymers (Marten, 2000). Additionally, the cristallinity and crystal morphology is dependent upon the processing conditions, and can change with time.

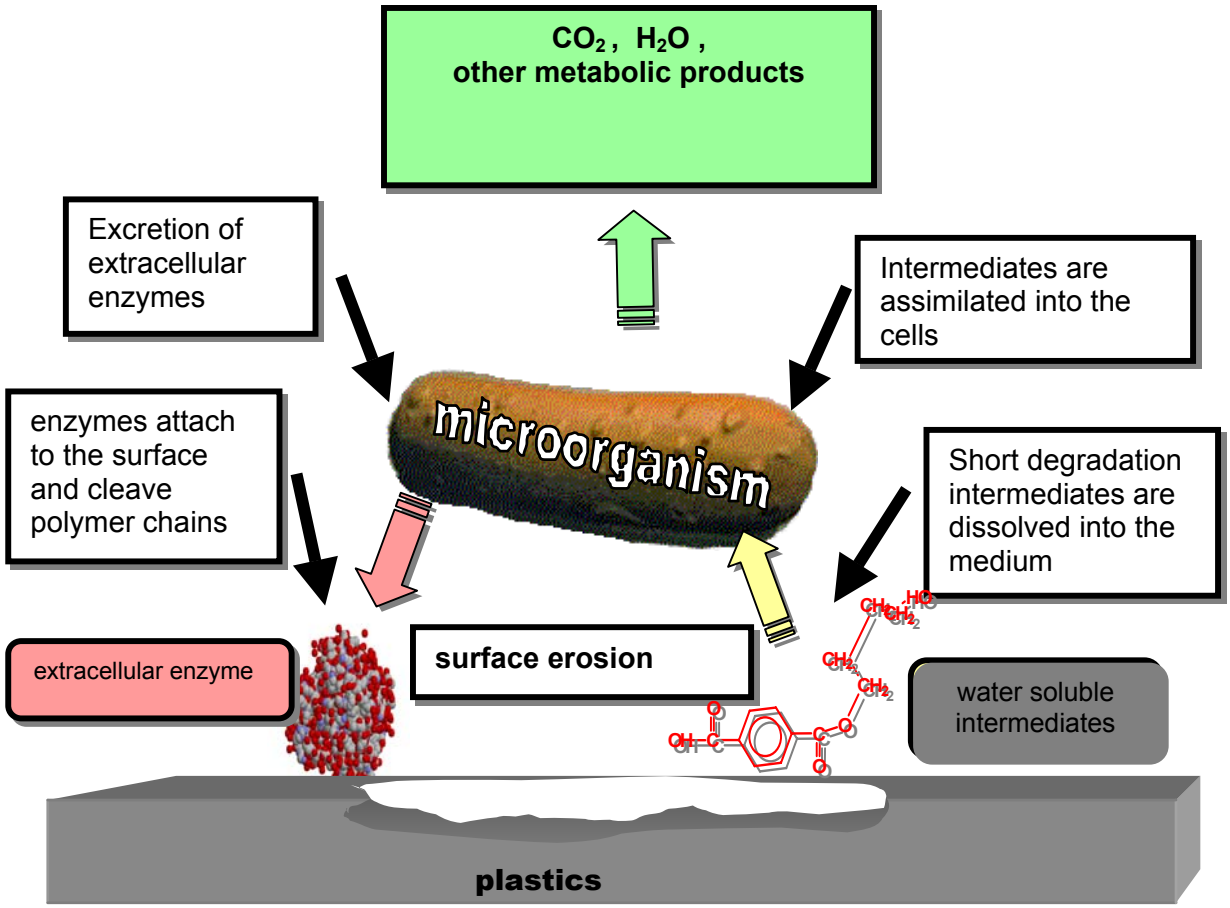


Fig. 2.1. General mechanism of plastics biodegradation under aerobic conditions after (Müller, 2003)

The standardized evaluation of biodegradable plastics should always be based on definitions, and biodegradation with regard to plastics actually means. Several different definitions have been published by national and international standardization bodies and organizations

2.2.2. Defining biodegradability

Unfortunately, the term biodegradation in connection with plastics has not been applied consistently for a long time, resulting in some confusion. Deterioration or a loss in physical integrity of a material is often mistaken for biodegradation. Biodegradation, however, is a natural and complex process of decomposition facilitated by biochemical mechanisms and successive mineralization of the polymer material.

There are a number of definitions describing biodegradability according to the fate of the polymer (Buchanan et al., 1993; Battersby et al., 1994)

- **Primary biodegradability** (or partial biodegradability) is the alteration in the chemical structure resulting in a loss of specific polymer properties.

- **Ultimate biodegradability** (or total biodegradability) deals with total mineralization and assimilation. The material is totally degraded by microorganisms with production of carbon dioxide (under aerobic conditions) or methane (under anaerobic conditions), water mineral salts and biomass (Augusta et al; 1992: Palmisano and Pettigrew. 1992).

However, two other definitions are also important for the biodegradation of organic compounds (Seal, 1991).

- **Ready biodegradable** is assessed in stringent tests which provide limited opportunity for biodegradation and acclimatization to occur.

- **Inherent biodegradable** is assessed in tests based on prolonged exposure of the test compound or other conditions favoring biodegradation. The degradation occurring under optimized conditions must not necessarily occur under normal test conditions.

In the present work the definitions of biodegradation as well as biodegradability for plastics according to the standardized test method DIN 54900-2 (1998) (Müller, 1994) are used, since they are the most stringent ones among the definitions laid down by ASTM, CEN, and ISO (Table 2.3):

Biodegradation: A process induced by biological activity which results through the change of the chemical structure of the material in naturally occurring metabolic products.

Biodegradability: A plastic material is biodegradable if all of its organic constituents are subject to complete biological degradation. The environmental conditions as well as the degradation rates are determined by standardized methods.

Table 2.3. General definitions of a biodegradable polymer (or plastics) proposed by standard Authorities and summarized by Calmon-Decriaud et al., (1998).

Standard Authorities	Biodegradable plastics
ISO 472-1988	A plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastic and the application in a period of time that determines its classification. The change in the chemical structure results from the actions of naturally occurring microorganisms.
ASTM sub-committee D20-96	A degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria and fungi.
Din 103.2-1993 German working group	A plastic material is called biodegradable if all its organic compounds undergo a complete biodegradation process. Environmental conditions and the rates of biodegradation are to be determined by standardized methods.
E.C.N (May 1993)	A degradable material in which degradation results from the action of microorganisms and ultimately the material is converted to water, carbon dioxide and/or methane and new cell biomass.
Japanese Biodegradable plastic is society (1994)	Polymeric materials which are changed into lower weight compounds where at least one step in the degradation process through metabolism in the presence of naturally occurring organisms.

In addition Pagga (1994) and Witt et al., (1997) reviewed important standardized test methods.

2. 3. Testing methods

1. General principles in testing biodegradable plastics

When testing the degradation phenomena of plastics in the environment, there is a general problem concerning the type of tests to be applied and the conclusions which can be drawn. In principle, tests can be subdivided into three categories: field tests; simulation tests; and laboratory tests (Fig. 2.2.).

Although field tests, such as burying plastics in soil, placing it in a lake or river, or performing a full-scale composting with the biodegradable plastic, represent the ideal practical environmental conditions, there are several serious disadvantages associated with these types of test. One problem is that environmental conditions such as temperature, pH, or humidity cannot be well controlled and secondly, the analytical opportunities to monitor the degradation process are limited. In most cases it is only possible to evaluate visible changes on the polymer specimen, or perhaps to determine disintegration by measuring weight loss. The latter approach is problematic if the material breaks into small fragments that must be quantitatively recovered from the soil, compost or water. The analysis of residues and intermediates is complicated by the complex and undefined environment. Since the pure physical disintegration of a plastic is not regarded as biodegradation in the sense of most definitions, these tests alone can never distinguish whether a material is biodegradable or not.

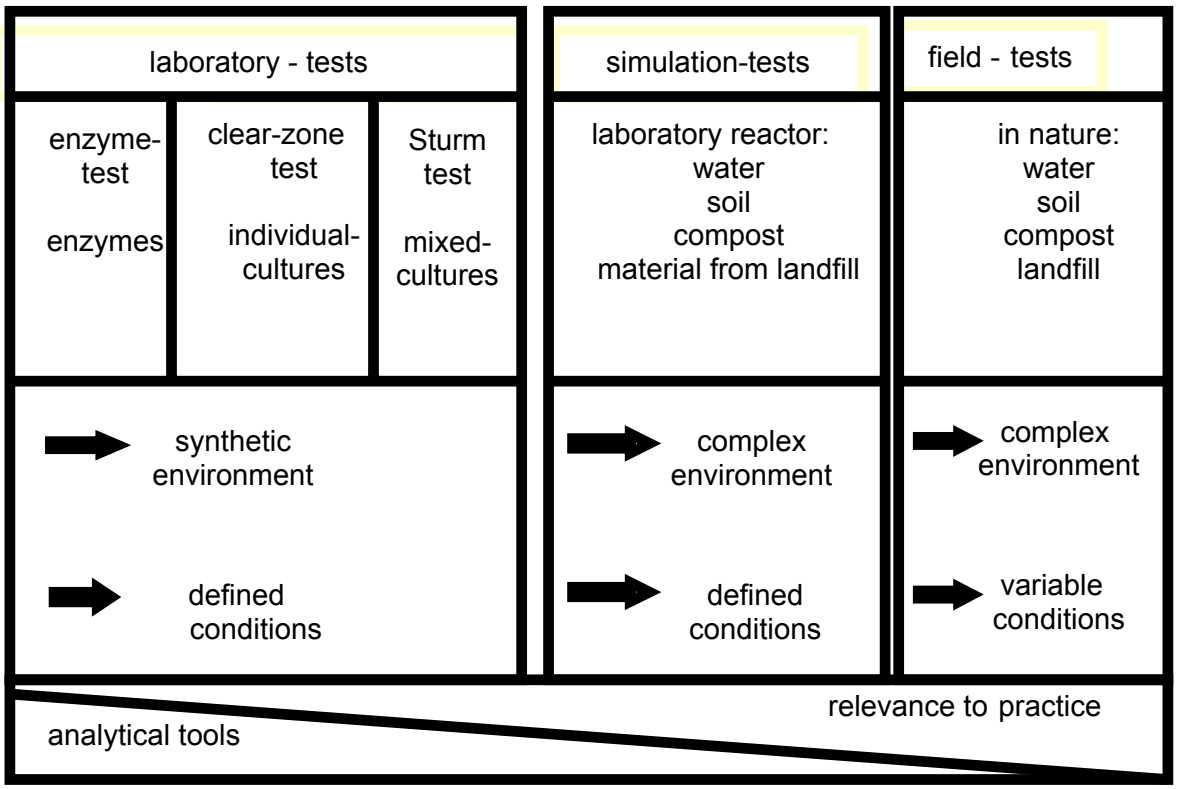


Fig.2.2. Schematic overview on tests for biodegradable plastics according to (Müller, 2003).

As an alternative to field tests, various simulation tests in the laboratory have been used to measure the biodegradation of plastics. Here, the degradation might take place in compost, soil or sea water placed in a controlled reactor in a laboratory . Although the environmental conditions are still very close to the field-test situation, the external parameters (temperature, pH, humidity, etc.) can be controlled and adjusted and the analytical tools available are better than for field tests (e.g. for analysis of residues and intermediates, determination of CO_2 evolution or O_2 consumption). Examples of such tests include the soil burial test (Pantke and Seal, 1990), the so-called "controlled composting test" (Pagga et al., 1995; Tosin et al., 1996; Degli-Innocenti et al., 1998; Ohtaki et al ., 1998; Tuominen et al., 2002), tests simulating landfills (McCartin et al., 1990) or aqueous aquarium tests (Püchner et al., 1995). On occasion, in order to reduce the time taken to conduct the tests, nutrients are added to increase the microbial activity and accelerate degradation.

The most reproducible biodegradation tests are the laboratory tests, where defined media are used (in most cases synthetic media) and which are inoculated with either a mixed microbial population (e.g., from waste water) or individual microbial strains which may have been especially screened for a particular polymer. In such tests, which may be optimized for the activity of the particular microorganisms used, polymers often exhibit a much higher degradation rate than it would be observed under natural conditions. This can be regarded as an advantage when studying the basic mechanisms of polymer biodegradation, but on the other hand in laboratory tests it is only possible to derive limited conclusions on the absolute degradation rate of plastics in a natural environment. However, for many systematic investigations these tests are widely used.

A move towards more reproducible and controlled degradation tests involves the use of systems where only those extracellular enzymes known to depolmerize a particular group of polymers are used. This method cannot be used to prove biodegradation in terms of metabolism by a microorganism, but the system is valuable when carrying out systematic investigations, for example on the correlation of polymer structure and biodegradability (Tokiwa and Suzuki, 1977; Vikman et al., 1995; Walter et al., 1995; Marten 2000). Besides reproducibility , the shortening of test duration and minimization of the material needed are crucial points when performing extended systematic investigation or when evaluating biodegradation as a part of industrial material development. Although degradation experiments in compost or soil may take up to one year to complete and tests with specially screened organisms may take only a few weeks, enzymatic degradation can be performed within hours to days. Recent approaches to improve degradability testing use very small polymer particles (nanoparticels) for enzymatic tests, in order to increase the surface area

available to the degrading enzymes. Using this technique, enzymatic degradation tests with polyesters can be performed within seconds (Gan et al., 1999; Welzel et al., 2002).

2. Analytical procedures for monitoring biodegradation

The analytical tools used to monitor the degradation process depends the aim of the investigation and the environment used. Some analytical methods will be discussed in more detail in the following sections.

a. Visual observations

The evaluation of visible changes in plastics can be performed in almost all tests. Effects used to describe degradation include roughening of the surface, formation of holes or cracks, defragmentation, change in color or formation of biofilms on the surface. These changes do not prove the presence of a biodegradation process in terms of metabolism, but the parameter of visual changes can be used as a first indication of any microbial attack. To obtain information about the degradation mechanism, more sophisticated observations can be made using either scanning electron microscopy (SEM) or atomic force microscopy (AFM) (Ikada, 1999).

b. Changes in mechanical properties and molar mass

As with visual observations, changes in material properties cannot be used to prove directly biodegradability with regard to a metabolism of the polymer material. However, changes in mechanical properties are often used when only minor changes in the mass of the test specimens are observed. Properties such as tensile strength are very sensitive to changes in the molar mass of polymers, which is also often taken as an indicator of degradation (Erlandsson et al., 1997). Whilst for an enzyme-induced depolymerization the material properties only change if a significant loss of mass is observed (the specimen become thinner because of the surface erosion process; the inner part of the material is not affected by the degradation process), for an abiotic degradation process (which often take place in the entire material and include the hydrolysis of polyesters or oxidation of polyethylene) the mechanical properties may changes significantly, though almost no loss of mass due to solubilization of degradation intermediates occurs at this stage. As a consequence, this type of measurement is often used for materials where an abiotic process is responsible for the first degradation step, e.g., chemical hydrolysis for poly(lactic acid) of oxidation for modified polyethylene (Breslin, 1993; Tsuji and Suzuyoshi, 2002).

c. Weight loss measurements: Determination of residual polymer

The mass loss of test specimens such as films or test bars is widely applied in degradation tests (especially in field- and simulation tests), although again no direct proof of biodegradation is obtained. Problems can arise with correct cleaning of the specimen, or if the material disintegrates excessively. In the latter case, the samples can be placed into small nets to facilitate recovery; this method is used in the full-scale composting procedure of DIN V 54900. A sieving analysis of the matrix surrounding the plastics samples allows a better quantitative of the disintegration characteristics.

For finely distributed polymer samples (e.g., powders) the decrease in residual polymer can be determined by an adequate separation or extraction technique (polymer separated from biomass or polymer extracted from soil or compost). By combining a structural analysis of the residual material and the low molecular-weight intermediates, detailed information regarding the degradation process can be obtained, especially if a defined synthetic test medium is used (Witt et al., 2001).

d. Clear zone formation

A very simple semi-quantitative method is so-called clear-zone test. This is an agar-plate test in which the polymer is dispersed as very fine particles within the synthetic medium agar; this results in the agar having an opaque appearance. After inoculation with microorganisms, the formation of a clear halo around the colony, indicates that the organisms are at least able to depolymerize the polymer, which is first step of biodegradation. This method is usually applied to screen organisms that can degrade a certain polymer (Nishida and Tokiwa, 1993; Abou-Zeid et al., 2001), but it can also be used to obtain semi-quantitative results by analyzing the growth of the clear zones (Augusta et al., 1993).

e. Others

Some other analytical methods to monitor biodegradation processes, especially for degradation experiments with depolymerizing enzymes include:

- Measuring the amount of dissolved carbon in the medium around the plastics by determining the dissolved organic carbon (DOC) content (Tokiwa et al., 1990).
- Decrease in optical density of small polymer particles dispersed in water (Jaeger et al., 1995).
- Analysis of the decrease in the particle size of small polymer particles using light scattering (Gan et al., 1999).
- Determination of free acids formed through enzymatic polyester cleavage by pH-stat titration (Walter et al., 1995; Marten, 2000; Welzel et al., 2002)

2.4. Isolation and selection of aerobic microorganisms for polyester biodegradation investigation.

The principle of enriching the desirable target organisms from the various kinds of organisms that coexist in a habitat was first developed by Winogradsky and Beijerink (Schlegel 1992; Brock and Madigan, 1991). For successful isolation of a given organism into pure culture, the organism generally must comprise a sufficiently high proportion of the mixed population. Enrichment methods are designed to achieve an increase in the relative numbers of a particular organism by favoring growth, survival (i.e. physiological competition), or its spatial separation from other members of the population. This method was previously successfully applied for the isolation of aerobic organisms capable of depolymerizing different polyesters such as poly (ε-caprolactone), poly(β-hydroxybutyrate), poly (ethylenesuccinate), poly (carbonate) and the synthetic aliphatic-aromatic copolyester BTA 40:60 (Benedict et al., 1983; Jendrossek et al., 1993B; Tansengco and Tokiwa, 1998; Suyama et al., 1998; Pranamuda et al., 1999; Kleeberg et al., 1999, and Abou Zeid et al., 2001). The selection criterion for polyester degradation is usually clear zone formation on agar plates containing the polyester of interest. The presence of clear zones proves the secretion of extracellular polyester depolymerizing enzymes by the selected target organism. The complete metabolization of the depolymerization products must be tested separately.

2.5. Biodegradation of aliphatic -aromatic polyester (BTA copolyester)

Aliphatic polyesters such as the natural polyhydroxyalkonates (e.g. PHB) and PCL as a synthetic aliphatic polyester are usually susceptible to microbial attack but lack in many properties important for application (Murphy et al., 1996, Oda et al., 1995,1997, Scherer et al., 1999, Ishigaki et al., 2000, Kim et al., 2000). In contrast, pure aromatic polyesters like poly(ethylene terephthalate), exhibited excellent material properties but proved to be almost resistant to microbial attack. With the intention of combining both, biodegradability and good material properties, copolyesters containing aliphatic and aromatic monomers were tested as biodegradable materials and recently, it was shown that such synthetic copolyesters containing aromatic constituents are also degraded by microorganisms. The combination of terephthalic acid (30 to 60 mol% of the acid components), adipic acid and 1,4-butandiol turned out to be the most appropriate combination both, with regard to the material properties (flexible films, melting point from 90-140°C) and biodegradability (such aliphatic-aromatic copolyesters were degraded in soil and compost in a range of 8 to 12 weeks (Witt et al., 1995). A number of thermophilic actinomycetes degrading aliphatic aromatic copolyesters have recently been isolated from compost, and taxonomically and identified as *Thermomonspora fusca*. *T. fusca* depolymerize the aliphatic-aromatic copolyesters very

rapidly, also in defined synthetic media, but do not metabolize significant amounts of the low-molecular weight substances formed (Kleeberg et al., 1998).
However, independent of the degradation mechanisms it has been proven that also aromatic oligomers formed during the depolymerization of the commercial copolyester Ecoflex® do not accumulate under conditions present during a composting process and no acute ecotoxic effect results from these intermediates (Müller et al., 2001, Witt et al., 2001).

2.6. The biological agent in biodegradation of polyester

The biological environment, i.e. the biologicl surroundings in which polymers are present, includes the biological agents responsible for the deterioration of polymeric substances. Biological agents such as bacteria, fungi and their enzymes consume a substance as a food source so that ist original form disapears. Under appropriate conditions of moisture, temperature and oxygen availability, biodegradation is a relatively rapid process.

2.6.1.Microorganisms

●Two types of microorganisms are of particular interest in the biodegradation of natural and synthetic polymers; these are bacteria and fungi where the bacteria include the general bacteria and actinomycetes. Many species or types of microorganisms are found broadly in nature after Chandra and Rustgi (1998)

● Fungi

Eumycetes, or true fungi, are microorganisms of particular importance in causing the degradation of materials. Fungi are nucleated, spore-forming, nonchlorophyllous organisms, which reproduce sexually as well as asexually; most of them possess filamentous, somatic structure, and cell walls of chitin and/or cellulose. More than 80000 species are known.

True fungi are present everywhere. Their importance as deteriorative agents is a result of the production of enzymes which breakdown nonliving substrates in order to supply nutrient materials present in polymer compositions. Certain environmental conditions are essential for optimum growth and degradative activity. These include an optimal ambient temperature, the presence of nutrient materials, and high humidity.

The group of test fungi that evolved for assay purposes in the field of natural polymers and that were further selected for their utility in assay procedures on synthetic polymers are taxonomically a very heterogeneous group, exhibiting no marked taxonomic similarities among them (for example based on morphology). Many of them were selected primarily because their reproduction spores are produced asexually and the variation associated with spores resulting from the fusion of sexual element is minimized. The test organisms cited are

also, for the most part, the selected organisms from a large number of isolations which have proved their capability for yielding reproducible results repetitively, over long periods of time, under laboratory conditions, and in synthetic or highly controlled and specific culture media.

● Bacteria

Bacteria can be single-cell rods, cocci, or spirilla; others are chain-like or filamentous. They can either be aerobic or anaerobic; in contrast, fungi are necessarily aerobic. Some bacteria are motile; bacteria are predominantly nonchlorophyllous. Their degradative action is also chiefly a result of enzyme production and resultant breakdown of the nonliving substrate in order to obtain nutrient materials.

Bacteria present in soil are important agents for material degradation. Particularly affected are cellulosic plant life, wood products and textiles subject to cellulytic degradation.

It is known that the fungi were classified under the the eukaryotic organisms and the bacteria was classified under the prokaryotic organisms. Table 2.4. summarizes the difference between the eukaryotic and prokaryotic organisms.

Biopolymers are formed in nature during the growth cycles of all orgainsms; hence, they are also referred to as natural polymers. Natural polymers are for instance cellulose, lignin or cutin. In nature, many microorganisms secrete different enzymes, which can degrade these natural polymers.

Many fungi and bacteria secrete cellulolytic enzymes which can degrade cellulose. These celulases include three principle types of enzymes: 1) endo-1,4-ß-gluconase, which randomly attck internal 1,4-ß-glucosidic bonds along the main chain to generate lower molecular weight polymers and oligomers (cellodextrins) 2) exo-1,4-ß-glucanases, which selectively hydrolyze terminal units on the chain; 3) 1,4- ß-glucosidases, which catalzye the hydrolysis of cellobiose and the higher, water-soluble cellodextrins to glucose to provide nutrients for the organisms (Schlegel 1992).

Lignin is a complex biopolymer occurring in plant material. The white-rot fungi and actinomycetes are known to be involved in the degradation of lignin (Crawford and Sutherland 1980, Kirk and Farrell 1987). Lignin peroxidase, mangenase-peroxidase, phenoloxidases and laccases are enzyme system that were involved in the biological degradation of lignin (Tien and Kirk 1983, Kirk and Farrell 1987).

Many plant pathogenic fungi and bacteria secrete cutinase, a hydrolase, which can degerade cutin (the structural polymer of the plant cuticle) (Purdy and Kollattukudy 1973, 1975, Ettinger et al., 1978, Lin and Kolattukuddy 1980, Köller and Parker 1989 Fett et al., 1992 and 1994, Fan and Köller 1998, Cunha et al., 2003).

Table 2.4. Differences between prokaryotic and eukaryotic cells after (Starr and Schmidt 1981)

Trait	Prokaryotic cells	Eukaryotic cells
Common size range (diameter)	0.3 to 2.5 µm	2 to 20 µm
Nucleus membrane bounded	No	Yes
Number of chromosome per nucleus	One	Generally more than one
Chromosome replication by mitosis	No	Yes
Histone proteins associated with nuclei	No	Yes
DNA location	In nucleoid –independent plasmids, neither of which are are membrane bounded	In nucleus and some organelles
Genetic recombination	Unidirectional transfer of DNA forms partial diploids	Fusion of gametes forms diploids that segregate by meiosis
Presence of nucleolus, Golgi apparatus, endoplasmic reticulum, lysosomes, mitochondria, microtubular systems	None	Usually present
Ribosome sedimentation coefficients	70S	80S (cytoplasmic); 70S (organellar)
Chloroplasts	None	Occur (in plants only)
Sterols in membranes	None (except sterols of exogenous origin in some mycoplasmas)	Often present
Cell wall and envelope components	Peptidoglycans, teichoic and lipoteichoic acids, lipoproteins, lipopolysaccharides	Cellulose and other polysaccharides (in plant only)
Organelles, bounded by unit membranes	No; organelles are relatively rare; when they occur, as in the cases of gas vesicles and chlorosomes (chlorobium vesicles), they are bounded by single-layer (nonunit) membranes (possible exception: the thylakoids of cyanobacteria)	Yes; many kinds of organelles
Flagella and cilia	No cilia; flagella are tripyl-threaded protein helices	Typical 9 + 2 arrangement in both flagella and cilia
Phagocytosis, pincytosis, cytoplasmic streaming, amoeboid movement	Absent	Often present

2.6.2. Polyester cleaving enzymes

Enzymes are, first and foremost catalysts. Structurally, enzymes are complex and highly specialized proteins, which produced by the cell, in order to catalyze specific types of chemical functions.

Enzymes are generally classified into six groups depending on the reaction they catalyse: oxidoreductases, transferases, hydrolases, lyases, isomerase's and ligases (Lehninger 1987, Uyama et al 2002). Polyester cleaving enzymes belong to the group of hydrolases, which catalyses the hydrolytic cleavage of the C-O and C-N- bonds. Hydrolases include lipases, esterases, PHB-depolymyerases and serine endopeptidases (Webb, 1992). A common feature for the serine hydrolases is the presence of a specific sequence Gly-X-Ser-X-Gly (Antonain 1988, Brady et al., 1990, Jendrossek et al., 1995, Schirmer et al., 1995, Arpigny et al., 1998). The catalytic mechanism of these enzymes is very similar and the catalytic center consists of a triade of serine, histidin and aspartate residues and other oxy-anion stabilizing rest groups (Kazlauskas and Bornscheuer 1998). Schirmer et al. (1995) demonstrated that serine from this sequence Gly-X-Ser-X-Gly- in the active center attacks the ester bond nucleophilically.

The biodegradation of polymers by microorganisms is catalyzed by extracellular, degradative enzymes that produce water soluble, low molecular weight products from the macromolecular substrates. These products are water soluble and can diffuse into the surrounding aqueous environment and are taken up by the cells of the microorganisms and used as nutrients. The extracellular degradative enzymes were produced by microorganisms degrading natural and synthetic polyesters**.**

1. Exo-and Endoenzyme

The enzymatic degradation of a polymer by hydrolysis is a two-step process, in which the enzyme first binds to the polymer substrate and then catalyzes a hydrolytic cleavage. This initial attack on the polymer can occur by two different mechanisms, known as exo- and endo-attack differing by both the locus on the polymer at which a bond is cleaved and the degradation–products that result.

The exo-attack occurs strictly at the polymer chain terminus, often with a preference for one chain end moiety over the other (e.g. the hydroxyl end of a hydroxyl-acid polyester rather than the carboxylic acid end), with exclusively small oligomers or monomers as the resulting products. On the contrary, the endo attack can, in principle, occur at any location along the length of the polymer chain and in that case a mixture of low molecular weight products results. Repeated endo cleavage, therefore, reduces the molecular weight of the polymer quickly, whereas repeated exo cleavage results in a rapid generation of small metabolizable

monomers, dimers, etc. These two degradation processes are illustrated schematically in Fig 2.3, where M represents both the internal repeating unit and the terminal units, the subscript represent the number of units in the product formed:

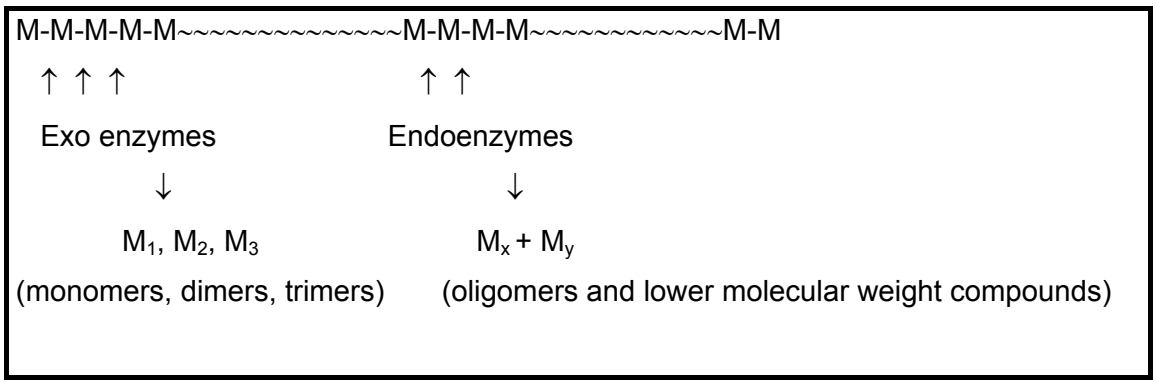


Fig 2.3. Exo- and endogen polymer cleavage (Lenz 1993)

In some systems, such as in the degradation of cellulose, both modes operate in synergy. (Huang 1989, Lenz 1993).

2. Carboxylic ester hydrolases

Two major classes of hydrolases are known: lipase (EC 3.1.1.1, triacylglycerol hydrolases) and true esterases (EC 3.1.1.3, carboxyl ester hydrolases) produced by both, procaryotic and eucaryotic microorganisms. These hydrolases show a very wide specificity for substrates such as triglycerides. According to Bornscheuer (2002), lipases can be distinguished from esterases by the phenomenon of interfacial activation, which is only observed for lipases. Whereas esterases obey classical Michaelis-Menten kinetics. Structure elucidation revealed that this interfacial activation is due to a hydrophobic domain (*lid*) covering the active site of lipase. Only in the presence of a hydrophobic phase interface the *lid* moves apart, making the active site accessible. Thus , lipases prefer water-insoluble substrates, typically triglycerids composed of long-chain fatty acids, whereas esterases preferentially hydrolyze water soluble esters (e.g. ethyl acetate) and cleave usually only triglycerides bearing fatty acids shorter than C_6. Both enzymes have been shown to be stable and active in organic solvents, but this feature is more pronounced with lipase. Table 2.5 show the difference between lipases and carboxyl esterases.

Table 2.5. Differences between lipases and carboxyl esterases after Bornscheuer, (2002)

Property	Lipase	Esterase
Preferred substrates	Triglycerides(long-chain),secondary alcohols	Simple esters, triglycerides (short-chain)
Interfacial activation/lid	Yes	No
Substrate hydrophobicity	High	High to low
Enantionselectivity	Usually high	High to low to zero
Solvent stability	High	High to low

The mechanism for ester hydrolysis or formation is essentially the same for both, lipases and esterases and is composed of four steps: first, the substrate is bound to the active serine, yielding in a tetrahedral intermediate stabilized by the catalytic His and Asp residues. Next, the alcohol is released and an acyl-enzyme complex is formed. Attack of a nucleophile (water in hydrolysis, alchohol or ester in (*trans*-)esterification) forms again a tetrahedral intermediate, which after resolution yields the product (an acid or an ester) and free enzyme (Bornscheuer and Kazlauskas 1999).

3. Results and Discussion

The most widely used screening method for polyester depolymerizing organisms is the so called “clear zone” method (Augusta et al., 1993; Jendrossek et al., 1993B, Kleeberg et al 1998, Abou Zeid et al., 2001). The extracellular hydrolyzing enzymes secreted by the target organism hydrolyze the suspended polyesters in the turbid agar medium into water soluble products thereby producing zones of clearance around the colony. The main advantage of this test is that it is generally fast, cheap and simple, and allows the simultaneous performance of a great number of parallel tests.

Depending on the different physical properties of the used polyesters, the development of special emulsification methods for the different polyesters was necessary. For PHBV, see Material & Methods, no special treatment was required and the polyester powder was directly mixed into the minimal agar medium prior to sterilization.

The synthetic polyesters applied in the present work agglomerated in the media, probably due to their low melting points and hydrophobic surfaces. Therefore the direct incorporation of polyester powders into the liquid medium was not possible. Casting of the polyesters dissolved in an adequate solvent on the surface of the agar plates resulted in turbid plates but prevented growth of organisms. Probably the hydrophobic film interrupted the nutritional transfer. However, suitable methods for the preparation were already described by other authors (see Material and Methods) and could be adapted to the polyesters used in this work.

3.1. Screening for polyester degrading isolates by applying the clear zone test method

The general screening- and isolation method of the polyesters degrading microorganism is shown in Fig (3.1). The isolation procedure consisted of three subsequent steps (see also chapter Material & Methods):

1) The microbial population containing the potential polyester degrading organisms was cultivated on emulsified mineral - salt -vitamin agar with BTA 45:55 (Ecoflex) or PCL (0.1% w/v).

2) Then, morphologically different colonies were identified as degrading species (for BTA-copolyester or PCL) via clear zone formation and selected for purification.

3) Positive strains were isolated by picking the colonies using sterile tooth picks or needles. The isolates were further purified on complex media (see Material&Methods) using the standard spatial streaking method on solid agar media plates for the bacterial isolates. Acidic complex medium such as malt extract agar medium pH 4.7 or addition of Ampicillin (800 mg/l) to the complex medium such as potato dextrose agar were used for the purification of fungal isolates.

4) The purified strains were tested for their ability to depolymerize the polyesters to estimate the degradation potential.

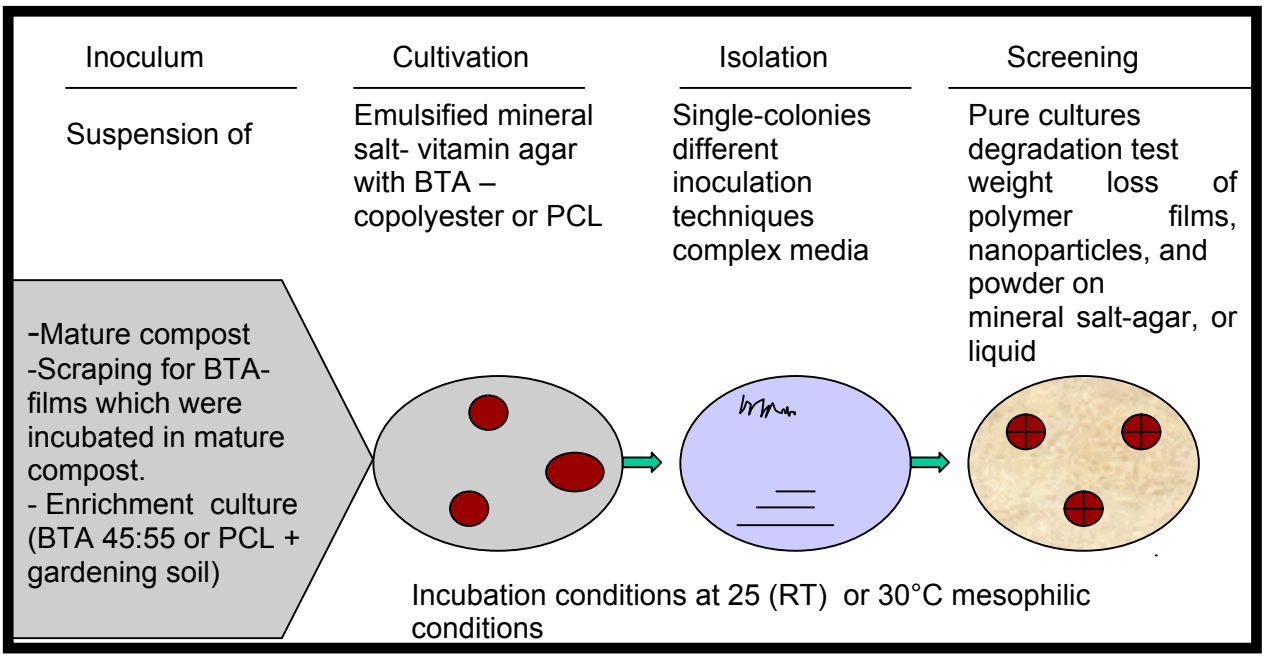


Fig. 3.1. Scheme for screening and isolation for microorganisms which are able to degrade the synthetic polymers.

From both microbial sources (mature compost at room temperature and an enrichment culture from gardening soil at 30°C) a total of 47 morphologically different BTA and PCL degrading isolates were obtained.

The results are compiled in Table 3.1. They give an overview of the total number, isolation source, and incubation temperature of the degrading isolates for the aliphatic-aromatic copolyester BTA and the aliphatic homopolyester PCL. A preliminary classification based on the morphology of the isolates revealed that the polyester degrading organisms belong to the group of fungi as well as to the group of bacteria. Within the later group actinomycetes play a dominating role.

Table 3.1. Numbers of microorganisms which were isolated from mature compost and gardening soil and their abilities to degrade the aliphatic-aromatic-copolyester and aliphatic homopolyester.

Type of polyesters	No. of isolates	Source of microorganisms					
		Mature compost			Gardening soil		
		Fungi	Actinomycetes	General bacteria	Fungi	Actinomycetes	General bacteria
PCL	24		10**	3**	5**	3**	3**
BTA copolyester	23 + 2[a)]	4*			6**	11**	2**

[a)] two isolates of *Gliocladium roseum* were obtained from BASF company, Ludwigshafen, Germany.

* at room temperature

** at 30°C

In both microbial sources polyester degrading isolates from the group of fungi (15 strains), actinomycetes (24 strains) and bacteria (8 strains) were obtained. Obviously, fungi and actinomycetes play an outstanding role in degrading the BTA copolyester and PCL under mesophilic conditions since the majority of strains belong to these groups. Also for several natural polymers, like chitin, celluloses, starch and lignocelluloses fungi and actinomycetes are known to be involved in their degradation (Crawford and Sutherland 1980, McCarthy and Cross 1984, McCarthy 1987, Kempf and Kutzner 1988). The dominant role of fungi in degrading polyesters at mesophilic conditions was already demonstrated earlier. Roßhaupten and Molitoris (1996) reported that different fungal isolates can degrade BTA 40:60 by using also the clear zone formation technique.

In contrast to mesophilic conditions only few polyester degrading fungi were found at thermophilic temperatures (Kleeberg et al., 1998). The authors isolated 61 thermophilic microorganisms degrading BTA-copolyester, among of those are 29 thermophilic

actinomycetes, 30 bacterial isolates, and only 2 fungal isolates. Under this conditions the degrading actinomycetes prevail on the degrading fungal isolates.
55 degrading synthetic homopolyester (PCL and SP4/6) and natural polyester isolates were isolated under anaerobic conditions, but no degrading BTA-copolyester (BTA 40:60) isolates were found under the same conditions Abou Zeid et al., (2001).

3.2. Estimation of degradation efficiency for the isolated strains

It was intended to comparatively evaluate the biodegradtion efficiency for the degrading isolates, and select the most efficient strains to be used for further investigation. The following procedures were applied for this comparison:

3.2.1. Clear zone formation

This test offer a number of advantages which described above. It can be used to obtain semi-quantitative results by analyzing the growth of clear zones (Augusta et al., 1993), but this technique was used only as preliminary test in the present work for the comparison between the isolates due to the reason, that fungi during their growth spread over the entire agar plate and thus, the clear zones formed can not appropriately used even as a semi-quantitative measure for the degradation potential of the fungi.

Therefore, the question remains open, how to discriminate between the degrading isolates and how to select the most efficient polyester degrading isolates for further investigation. With regard to the application of the isolated strains for improved degradation tests of synthetic polyesters, i.e. the estimation of weight loss for polymer films on agar plates, was used.

3.2.2. Determination of weight loss of polymer films on agar plates at 30°C

Usually, enzymatic degradation of plastics is a surface erosion process, because enzymes are not able to penetrate the bulk polymer. Thus, the rate of weight loss can be directly used to measure the enzymatic cleavage of the polymer chains.

The degradation potential of all isolates was quantified (expressed as weight loss (mg), % degradation, and degradation rate per surface area [mg/(week cm²)] for polymer films on agar plates after 4 weeks incubation at 30°C.

3.2.2.1. Polyesters degrading fungal isolates

All fungal isolates grew on mineral salts agar (MSV) plates containing polymer films (BTA 45:55 (Ecoflex), BTA 40:60 and PCL, respectively)) as a sole carbon source. According to the morphological characteristics and microscopical examination, the fungal isolates were divided into different subgroups, while each subgroup include the similar strains in it morphological and microscopical appearance. Fig 3.2 and Table 3.2 shows the experimental results of the quantitative determination of weight loss (mg) of BTA-copolyester (BTA 45:55 and BTA 40:60) and PCL films with all fungal isolates. In most cases PCL films were degraded much faster than the BTA copolyester (BTA 40:60 and BTA 45:55 (Ecoflex)) films which was expected from earlier investigations (Abou Zeid et al., 2001). However BTA 40:60 films were degraded as fast as PCL films by three fungal strains (EB1, EB6 and EB8). BTA 45:55 (Ecoflex) was disintegrated more slowly than the two other polyesters by all isolates. Polyesters with such a large aromatic compound fraction have also been shown to degrade slowly in composting tests (Witt et al., 1995).

The degradation potential obtained for BTA copolyester by three subgroups (1, 3 and 5) was higher than those of other subgroups (2 and 4). On the other hand the degradation potential of three groups (2, 3 and 5) obtained for PCL films was higher than for the other two subgroups (1 and 4). The results were compared with those obtained with noninoculated films (noninoculated controls) incubated in the respective media. The noninoculated controls showed no weight loss.

From 14 screened isolates 4 fungal strains, namely EB1, EB10, EB14 and EB19 as well as two strains from *Gliocladium roseum* (G1 and G2) were selected as they comprised the highest polyesters depolymerization potentials and were used for further investigations and taxonomic identification.

Table 3.2. Degradation potential of fungal strains measured as weight loss [mg] for BTA copolyester and PCL films and the trend of biodegradability.

(Medium: MSV-agar (mineral salt agar), Temperature: 30°C, Incubation time: 4 weeks, Polymer films: diameter: 25 mm, surface area: 4.91cm², initial weight of the films: BTA 45:55 (Ecoflex) 26.83 -32.8 mg, PCL: 41.1 – 42,6 mg, BTA 40:60: 70.5 – 80.4 mg, Thickness of films: BTA 45:55 : 60 µm, BTA 40:60 : 120 µm, PCL : 75 µm, n = 3 parallel films per test, Inoculum: 1 disc (5 mm in diameter) from complex medium bearing the fungal mycelium.

Fungal sub-groups	Code number of the fungal isolates	Range of weight loss [mg] for polymer films			Trend of biodegradability
		BTA-copolyester		PCL	
		BTA 45:55 (Ecoflex)	BTA 40:60		
Sub group 1	EB1, EB6, EB8	8.6-12.2	21- 33.3	16.9 - 30.9	BTA 40:60>PCL> BTA 45:55 (Ecoflex).
Sub group 2	EB9, EB10, EB11, EB12 EB13	2.7 - 5. 6	9.7 - 17.6	38.5 - 41.2	PCL>BTA 40:60> BTA 45:55 (Ecoflex).
Sub group 3	EB14	9.9	25.1	41.1	PCL>BTA 40:60> BTA 45:55 (Ecoflex).
Sub group 4	EB15, EB16EB17, EB18, EB19	2.6 - 6.5	5.7 – 7	14 - 18.3	PCL>BTA 40:60> BTA 45:55 (Ecoflex).
Sub group 5	**G1, G2	4 - 8.2	8.4 - 27.4	14.9 - 40.6	PCL>BTA 40:60> BTA 45:55 (Ecoflex).

**Two isolates for *Gliocladium roseum* (obtained from BASF AG, Ludwigshafen)

The sixth subgroup include one strain designated as EB2T but this strain would not be tested at 30°C because it does not grew at 30°C.

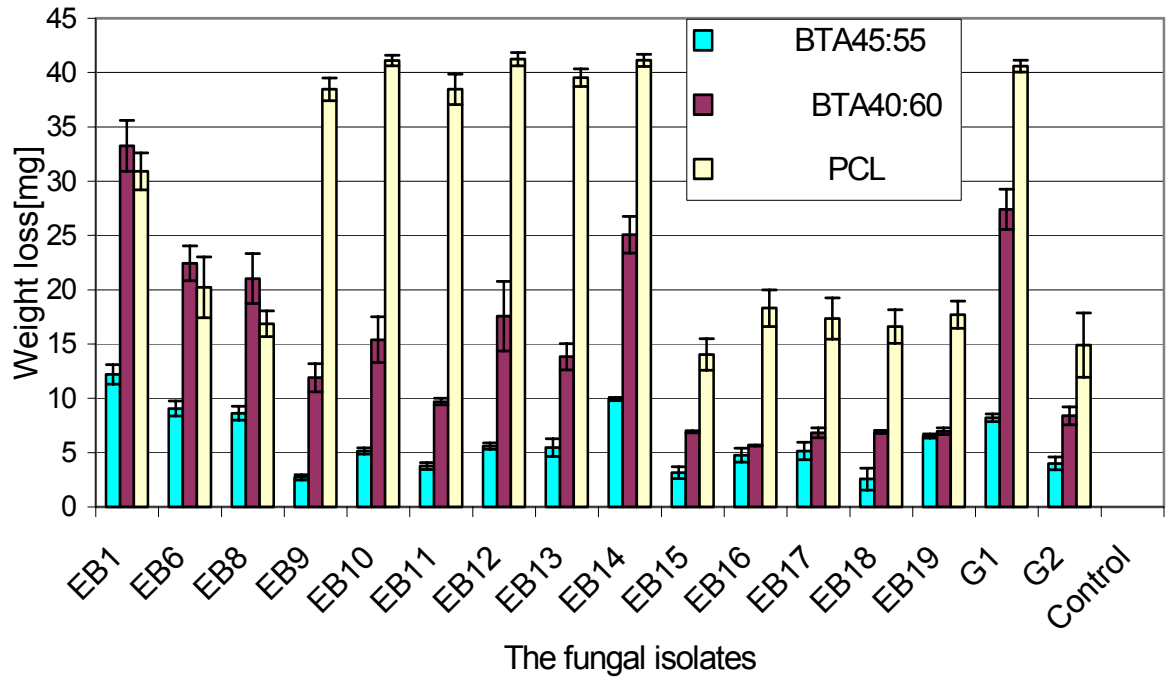


Fig.3.2. Determination of weight loss of BTA- copolyester and PCL films after 4 weeks at 30°C with the fungal isolates.

Medium: MSV-agar (mineral salt agar), Temperature: 30°C, Incubation time: 4 weeks, Polymer films: diameter: 25 mm, surface area: 4.91cm², initial weight of the films: BTA 45:55 (Ecoflex) 26.83 -32.8 mg, PCL: 41.1 – 42,6 mg, BTA 40:60: 70.5 – 80.4 mg, Thickness of films: BTA45:55 : 60 µm, BTA 40:60 : 120 µm, PCL : 75 µm, n = 3 films per test, Inoculum: 1 disc (5 mm in diameter) from complex medium bearing the fungal mycelium.

3.2.2.2. Polyesters degrading bacterial isolates

As shown for the fungal isolates , the polyester degrading bacteria were classified according to their degradation potential and morphological appearance. Generally the bacterial isolates could be assigned to actinomycetes and general bacterial. Within the actinomycetes the sub groups could be classified according to their morphological appearance and visible changes (colour) of the polymer films during the degradation test. In the sub group 1 the colour of the polyester films changed from white to red and in the sub group 2 from white to dark-brown due to the pigments which were produced by the microorganisms. Fig 3.3 and Fig 3.4 show examples for these visual observation with isolate E11 and Act32 as respentative typical strains for sub group 1 and sub group 2, respectively.

Fig 3.5 and Table 3.3 show the experimental results of the degradation potential for BTA copoylester and PCL films by the isolated bacterial strains. Of the polyester based materials tested, the aliphatic polyester PCL exhibited the highest degradation potential, while the aliphatic-aromatic copolyester (BTA 45:55 and BTA 40:60) was degraded slowly by most strains. Tokiwa and Suzuki (1981) reported that the biodegradability of the polyester decreased with increasing melting point (T_m). Unfortunately, the melting point (T_m) of PCL are low to permit their use in many applications.

As mentioned above applying fungal strains BTA 45:55 (Ecoflex) was disintegrated more slowly than BTA 40:60 by most strains due to increasing the ratio from aromatic compound. Tokiwa and Suzuki (1981) reported that the susceptibility to hydrolysis decreased with increase in aromatic polyester content. The highest remarkable degradation obtained for BTA copolyester and PCL films with strains subgroup 1 was higher than those of strains for the two other sub groups (2 and 3).

Within strains sub group2, 2 of those (strains H1 and Act21) degrade BTA 40:60 faster than PCL; Also 2 strains (strain Act23 and Act17) from the same group can degrade BTA copolyester ≈ PCL.

Among the 8 general bacterial isolates which were all aerobically growing rods mainly without endospore formation, only one strain was unable to disintegrate whether the copolyester nor PCL films.

Out of the 32 bacterial degrading isolates (actinomycetes general bacteria), 8 strains (E11, Act3, Act22, Act4, Act23, Act32, E12 and Pop) with high degradation activities for the BTA copolyester and PCL were selected for further investigations.

Table 3.3. Comparison of degradation of polyesters measured as weight loss of polymer films by the bacterial strains on agar plates.

(Medium: Mineral salt Vitamin (MSV) agar, Incubation time: 4 weeks, Polymer films ∅=25 mm, the surface area: 9.82 cm², pre-weight and thick of the films : BTA 45:55 (Ecoflex): 26.13 – 33.13, mg, 60 µm, BTA 40:60: 59.7 – 81.03 mg, 120 µm, PCL: 38.43- 42.4 mg, 75 µm, Inoculum with degrading bacterial isolates: 200 µl/film (10^7 CFU/ml) n:3 parallel films per test)

Bacterial sub-groups	Code number of the bacterial isolates	Range of weight loss [mg] for polymer films			Trend of biodegradability
		BTA-copolyester		PCL	
		BTA 45:55 (Ecoflex)	BTA 40:60		
Sub group 1*	E11, Act1, Act2, Act3, Act5, Act7, Act8, Act11, Act13,Act22, Act24 Act31	3.6 – 11.7	5.5 – 11.5	18 – 36.5	PCL>BTA 40:60> BTA 45:55 (Ecoflex).
Sub group 2*	Act4, Act6,Act12, Act16, Act17, Act21,Act23, Act32, Act33, Act34, Act35, and H1	1.7 – 4.4	2.2 - 6.5	0.6 – 17	1) PCL>BTA 40:60> BTA 45:55 (Ecoflex) 2)BTA 40:60> BTA 45:55>PCL 3) BTA copolyester ≈ PCL
Sub group3**	E12, PAB, Pd, Pc, Pop, Pf, Pov7 Pxw	0 – 4.7	0 – 9.3	0 – 26.1	PCL>BTA 40:60> BTA 4 5:55 (Ecoflex).

* Both sub groups are actinomycetes

** General bacteria

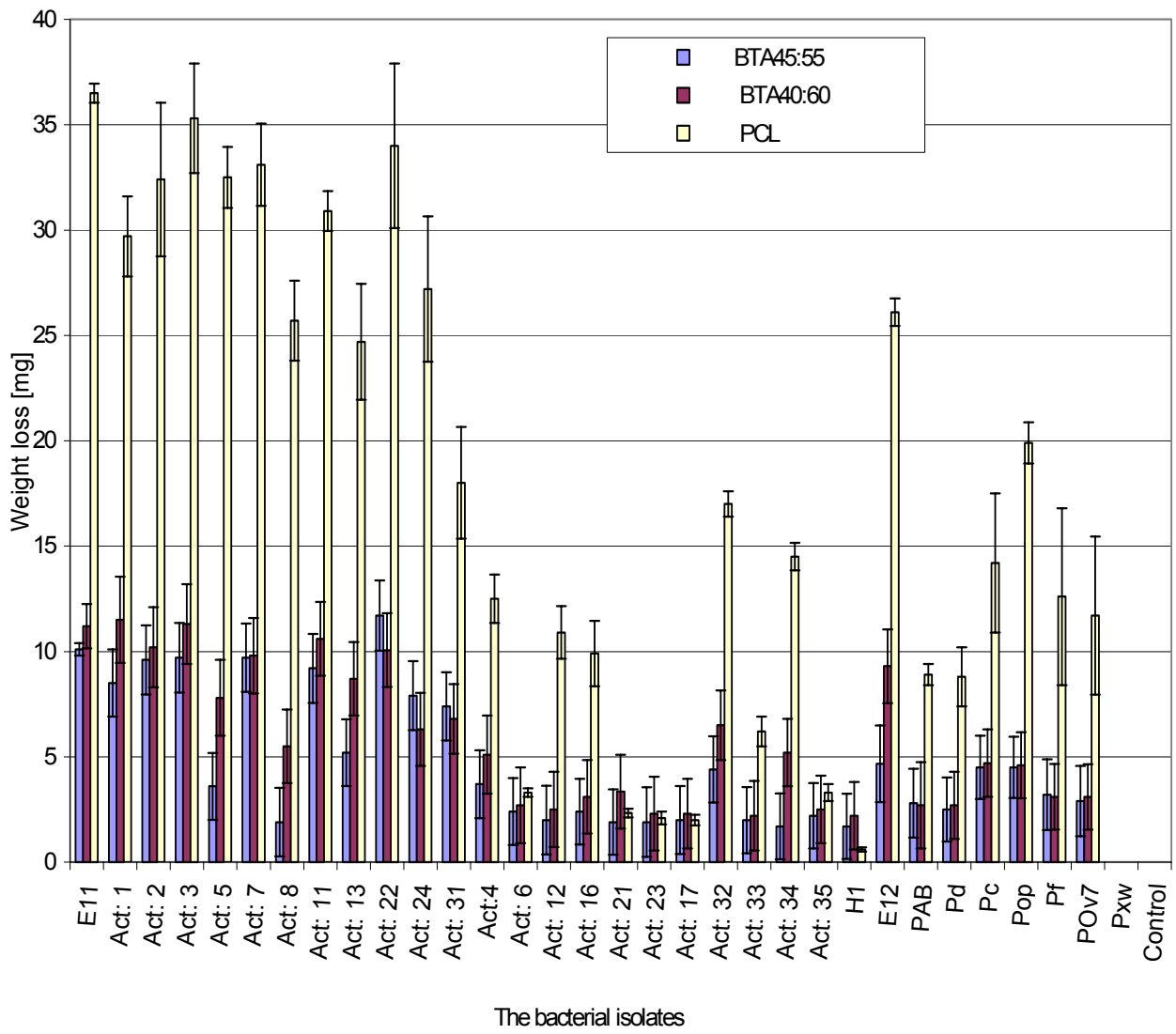
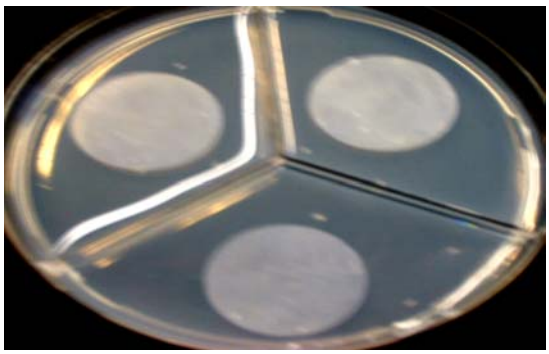
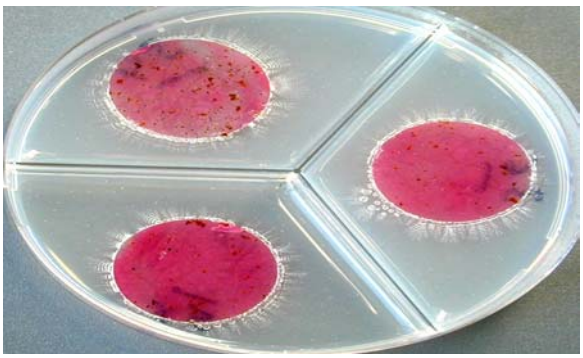


Fig. 3.5. Determination of weight loss (mg) with the bacterial isolates for BTA- copolyester and PCL films after 4 weeks at 30°C.

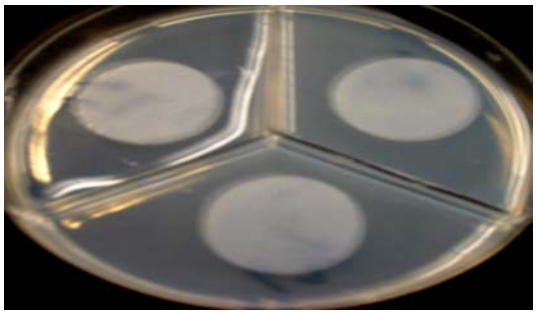
Medium: Mineral salt Vitamin (MSV) agar, Incubation time: 4 weeks, Polymer films ∅=25 mm, the surface area: 9.82 cm², pre-weight and thick of the films : BTA 45:55 (Ecoflex): 26.13 – 33.13, mg, 60 µm, BTA 40:60: 59.7 – 81.03 mg, 120 µm, PCL: 38.43- 42.4 mg, 75 µm, Inoculum with degrading bacterial isolates: 200 µl/film (10^7 CFU/ml) n:3 films per test



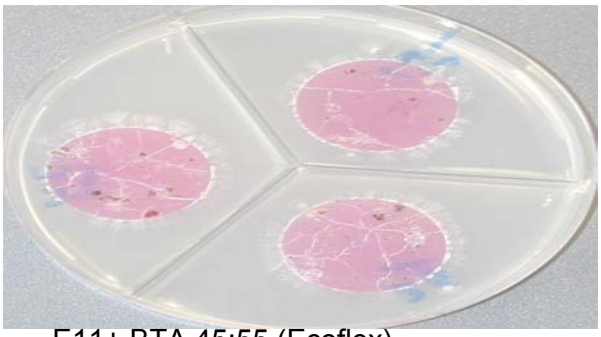
Control of BTA 40:60



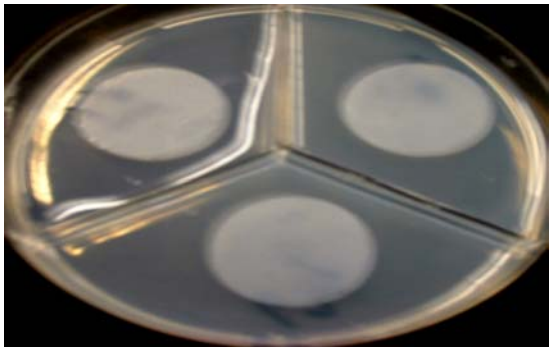
E11+BTA 40:60



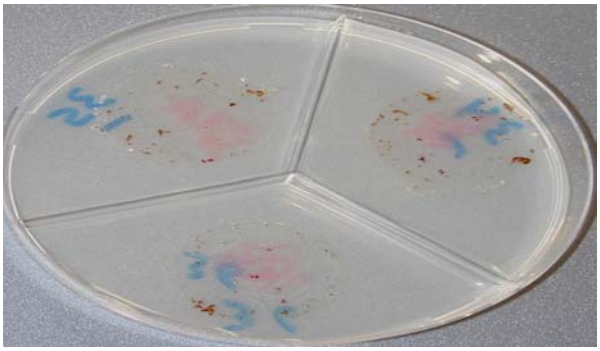
Control of BTA 45:55 (Ecoflex)



E11+ BTA 45:55 (Ecoflex)



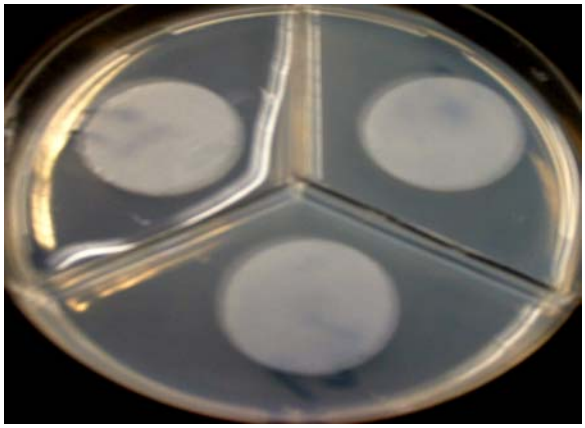
Control of PCL films



E11+PCL films

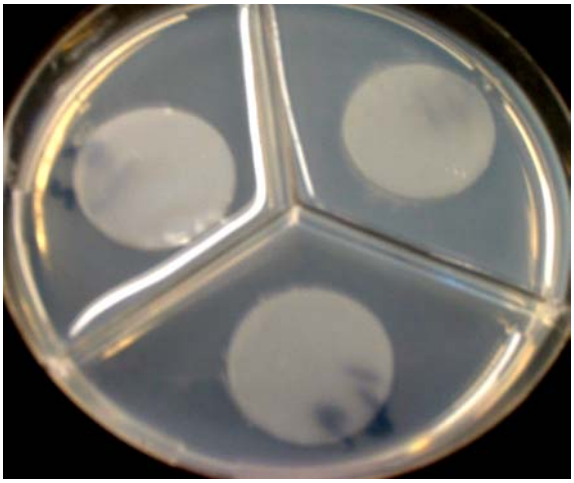
Fig. 3.3. The visual observation during degradation for BTA 45:55 (Ecoflex),BTA 40:60 and PCL films with the strain E11 on agar plates at 30°C.

Act32 + PCL films



Control PCL films (noninoculated)

Act32 + BTA 40:60 films



Control BTA 40:60 films (noninoculated)

Fig. 3.4. Visual observation during the degradation of BTA 40:60 and PCL films with strain Act32 on agar plates at 30°C.

It was observed with most isolates that PCL as aliphatic homopolyester was degraded faster than aliphatic-aromatic BTA copolyester. This behaviour was expected from data found in the literature. Marten (2000) postulated for instance that the reduced flexibility of the copolyester chains, which correlates with the relative high melting points of the copolyesters, hinders the polymer chains to adjust into the active site of the hydrolyzing enzymes.

An unusual degradation behaviour was found for the fungal isolates EB1, EB6 and EB8. Here BTA 40:60 was degraded as fast as the aliphatic polyester PCL. It can be anticipated, that these organisms and their extracellular enzymes prefer hydrophobic surfaces as offered by the BTA-

copoylesters. Kleeberg et al., (1998) reported earlier that BTA 40:60 was degraded much faster than aliphatic polyesters like Bionolle, Bayer Tir 1874 and SP313 under thermophilic conditions by two strains from *Thermomonspora fusca*.

3.3. Determination of the polyester substrate specificity of the selected isolates .

Among 47 polyester degrading isolates, 15 strains exhibit relative high degradation activities for the BTA copolyester and PCL films. These 15 isolates (7 fungi, 6 actinomycetes and 2 general bacteria) were selected for further investigation.

The question arose now if these BTA and PCL degrading isolates attack other polyesters, too. Therefore, the aim of the following investigations was to characterize the substrate specificity and depolymerizing potential of the particular organisms for different other polyesters. The selected microorganisms were tested with SP 4/6 as another synthetic aliphatic homopolyester and PHBV as natural copolyester by clear zone formation as preliminary test. The experiments were carried out at 30°C after 1 weeks with most isolates but only at 15°C with strain EB2T because this isolate did not grow at 30°C. The results in Table 3.4 show that, as expected, all isolates can degrade all synthetic polyesters under investigation (BTA copolyester, PCL and SP 4/6). In contrast not all of the isolates were able to attack the natural polyester PHBV. While none of the two general bacteria could attack PHBV, 4 out of 7 fungal – isolates and all actinomycetes were able to degrade the natural copolyester. This shows that especially actinomycetes and fungi are versatile in their ability to degrade the polymers under investigation.

Table 3.4. Substrate spectrum for the selected polyester degrading isolates characterized by clear zone formation on MSV-agar containing the polyesters (0.1% w/v). (Incubation time 1 week, Inoculum: 1 disc from complete medium bearing the fungal mycelium for fungal isolates, 1 loopful from bacterial growth). Incubation temperature: 30°C for 1 week with most isolates but only at 15°C with strain EB2T after 1 week.

The isolates		Aliphatic -aromatic copolyester (BTA) (BTA 40:60 and BTA 45:55 (Ecoflex))	Synthetic aliphatic homopolyester		PHBV as natural copolyester
			PCL	SP 4/6	
Fungi	EB1	+	+	+	-
	EB10	+	+	+	+
	EB14	+	+	+	-
	EB19	+	+	+	+
	G1	+	+	+	-
	G2	+	+	+	+
	EB2T*	+	+	+	-
Actinomycetes	E11	+	+	+	+
	Act3	+	+	+	+
	Act22	+	+	+	+
	Act4	+	+	+	+
	Act23	+	+	+	+
	Act32	+	+	+	+
General bacteria	E12	+	+	+	-
	Pop	+	+	+	-

+ clear zone - no clear zone

*EB2T was tested at 15°C because this isolate did not grow at 30°C.

Additionally to the qualitative clear-zone tests, the degradation potential of the isolates was characterized via weight loss measurements of polymer films on agar plates with the same isolates. The experiments were carried out at 30°C after 4 weeks with most isolates but only at 15°C after 6 weeks with strain EB2T because this isolate did not grow at 30°C. The degradation potential obtained for SP 4/6 and PHBV was compared with the degradation potential for BTA 45:55 (Ecoflex), BTA 40:60 and PCL which obtained it with most isolates at 30°C after 4 weeks in chapter 3.2.2.1 and 3.2.2.2 and with strain EB2T in chapter 3.7 at 15°C after 6 weeks. The results in Fig.3.6 and Table 3.5 summarize the differences of weight loss for polymer films. As expected the synthetic aliphatic and natural polyesters exhibited the highest weight losses in most cases, while the BTA copolyester was degraded slowly by most isolates. The general

trend of the biodegradability was PHBV>SP 4/6>PCL>BTA 40:60>BTA 45:55, however the fungal strain EB1 degraded BTA 40:60 as fast as PCL. In most cases, the degradation potential obtained with strains E12 and Pop as general bacteria was lower than those of fungal and actinomycetes strains. The experimental results were compared with the noninoculated (control), which showed no weight loss.

The production of pigments during degradation which was observed with strains sub group 1 from the actinomycetes (see Fig.3.6) was not observed with PHBV films.

Table 3.5. Determination of weight loss [mg] for different polyester films on agar plates by degrading isolates.

Polymer films (diameter, 2.5 cm; degradation surface area 4.91cm² with fungal isolates and 9.82 cm² with bacterial isolates) were incubated at 30°C for 4 weeks with most isolates but only at 15°C for 6 weeks with EB2T on MSV agar. Each series was carried out with triplicate samples. Inoculum: 1 disc (5 mm in diameter) from complex medium bearing the fungal growth per film, 200 µl/film for bacterial isolates (10^7 CFU/ml)

The isolates		Weight loss of synthetic polyesters [mg]				Weight loss of PHBV as natural copolyester [mg]
		Aliphatic aromatic copolyester (BTA)		Synthetic aliphatic homopolyester		
		BTA 45:55** (Ecoflex)	BTA 40:60**	PCL**	SP 4/6	
Fungi	EB1*	12.2	33.3	30.9	59.8	-
	EB10	5.2	15.4	41.1	56.8	75.9
	EB14*	9.9	25.1	41.1	44.4	-
	EB19	6.5	7	17.7	48.4	103.8
	G1*	8.2	27.4	40.6	42.9	-
	G2	4	8.4	14.9	20.1	46.5
	EB2T*	10.5	13	26.4	27.4	-
Actinomycetes	E11	10.1	11.2	36.5	50.6	61.5
	Act3	9.7	11.3	35.3	41.7	68.4
	Act22	11.7	10.1	34	53.4	67.8
	Act4	3.7	5.1	12.5	36.7	42.7
	Act23	1.9	2.3	2.1	25.2	57.7
	Act32	4.4	6.5	17	22	66.4
General bacteria	E12*	4.7	9.3	26.1	31.9	-
	Pop*	4.5	4.6	19.9	25.6	-

*these isolates were not tested with PHBV, because they did not form any clear zones on PHBV-MSV-agar.

**the same results which obtained it with the most isolates at 30°C after 4 weeks in chapters 3.2.2.1 and 3.2.2.2 but with strain EB2T at 15°C after 6 weeks in chapter 3.7.

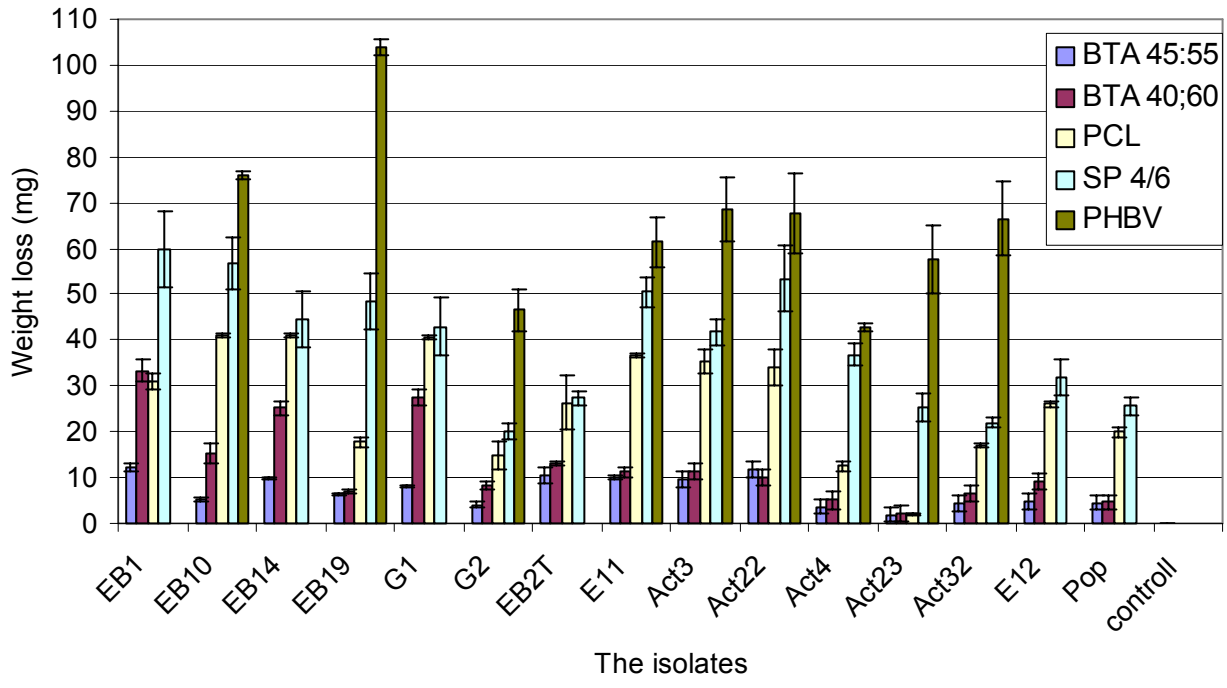


Fig.3.6. Determination of weight loss (mg) for different polyesters films by the selected isolates. Polymer films (diameter, 2.5 cm; degradation surface area 4.91cm² with fungal isolates and 9.82 cm² with bacterial isolates) were incubated at 30°C for 4 weeks on MSV agar with most isolates but only with EB2T at 15°C for 6 weeks . Each series was carried out with triplicate samples. Inoculum: 1 disc (5mm in diameter) from complex medium bearing the fungal mycelium per film, 200 µl/film for bacterial isolates (10^7 CFU/ml)

Abou-Zeid et al., (2001) demonstrated that PHBV and PCL films were degraded by strains *Clostridium botulinum* and *Clostridium acetobutylicum* under anaerobic conditions at 35°C and 37°C, respectively. By comparison the degradation rate for the two polymer films under anaerobic conditions with the same strains and mesophilic condition in the present work, the degradation rate under mesophilic conditions was about 25-61 fold for PHBV films and 4-73 fold for PCL films higher than under anaerobic conditions.

According to the observed substrate specificity, the degrading strains could be classified into two separate groups.

● **Strains degrading only synthetic polyesters**

The synthetic polyesters degrading strains are specialized to degrade only the synthetic polyester including BTA-copolyester (BTA 45:55 (Ecoflex) and BTA 40:60 PCL, SP 4/6) and cannot attack the a natural copolyester like PHBV. This group includes the fungal isolates (EB1, EB2T, EB14, and G1) and the two general bacterial isolates (E12 and Pop).

● **Strains degrading natural and synthetic polyesters**

These strains show a wider substrate spectrum degrade the synthetic and natural polyesters, as well. The group includes 3 fungal isolates (EB10, EB19, G2) and 6 actinomycetes isolates (E11, Act3, Act22, Act4, Act23, Act32).

The ability of individual microbial strains to degrade the synthetic and natural polyesters was investigated earlier by many authors. Pranamuda et al., (1995) showed that actinomycete strain HAT-6 can degrade Polyhydroxybutyrate, Polycaprolactone, and Bionolle. Mergaert and Swing (1996) isolated 171 polymer-degrading isolates, from these isolates actinomycetes and fungal isolates were able to degrade for polyhydroxybutyrate, Bionolle and polycaprolactone. Kleeberg et al., (1998) isolated thermophilic actinomycetes strains degrades BTA-copolyester as synthetic polyester and PHB as natural polyester and other polyester and this strains was identified as *Thermomonspora fusca*.

The question has to be posed, what is the reason for the different substrate specificities. It has been reported in the literature, that at least different enzyme systems are involved in the aerobic degradation of the different polyesters. These different kinds of enzymes are PHB deplomyerases, lipases and cutinase. Aerobic PHB depolymerases show no significant lipase activity or attack synthetic polyesters (Jaeger et al., 1995). However, several lipases hydrolyze synthetic polyesters such as PCL, but on the other hand, cannot hydrolyze PHB (Pranumuda et al., 1995). The third group of enzymes, cutinases are serine hydrolases for primary alcohol esters (Kazlauskas 1994; Svendsen 1994, Murphy et al., 1996) which depolmerize cutin specifically, and PCL probably due to structural similarities between its depolymerization products and those another structurally similar to cutin.

Therefore, the behaviour of the degrading microorganisms in the both classes shown above can be explained as follows: In the first group, where the organisms are specialized to degrade the synthetic polyesters the organisms produce exclusively lipase or cutinase enzymes. On the other hand the organisms which degrade synthetic and natural polyesters as well, probably produce at least two different enzymes, which are depolymerase and either lipase or cutinase.

3.4. Optimization of the cultivation conditions for the polyesters degrading microorganisms

Normally, the pH and temperature influence the growth of microorganisms and hence, these factors will influence also the degradation process of the polyesters. The question is now, what are the optimal conditions (pH and temperature) for the growth of the polyester degrading isolates?. To determine the optimal growth conditions for all isolates, in a first step glucose was used as carbon source instead of polyesters in mineral salt vitamin liquid medium.

In preliminary tests glucose was used with different concentration to answer the question, which is the optimal concentration of glucose for the growth of the fungal and bacterial isolates. This glucose concentration then will be used in the further investigation. The fungal growth was determined by mycelium dry weight after 1 week incubation at 30°C for strains EB1, EB10, EB14, EB19 and at 15°C for strain EB2T (see Material and Methods) and the bacterial growth was determined by measuring of the intracellular protein after 3-4 days incubation (3 days for general bacteria and 4 days for actinomycetes) at 30°C and 160 rpm (see Material and Methods).

From the results shown in Fig 9.1 and Fig. 9.2 optimal growth was determined for the fungal isolated at a glucose concentration of 15-20 g/l and for the bacterial strains of 8 g/l.

These glucose concentrations (15 - 20 g/l for fungal isolates) and 8 g/l for the bacterial strains) were used in the further investigation to determine the optimal pH and temperature for the microbial growth.

3.4.1. Polyester degrading fungal isolates

3.4.1.1. Optimal pH

The influence of pH on biomass yield of the selected fungal isolates is shown in Fig 3.7. Generally, the optimal pH was 7 for all the isolates. Among the seven fungal isolates, four (EB10, EB14, EB19, EB2T) grew at a quite wide pH range from 4 to 8. The G2 isolate grew in a range from pH4 to pH7, but exhibited no growth at pH 8. Surprisingly the isolates EB1 and G1 only grew exactly at pH 7, despite both isolates exhibited remarkable degradation activities for the synthetic polyesters.

It is known that the fungal organisms can grow on a wide range from pH 4 (acidic) to pH 7 (neutral). This variation is very useful to use these isolates in degradation test in different environments at different pH. Therefore, it can expect that these isolates can tolerate the pH changes during the degradation process thereby increase the degradation potential for these isolates.

An unusual growth of two isolates (EB1 and G1) was observed in as far as they grew only on pH 7 and therefore use of these isolates in degradation test is under defined conditions. Therefore, one can expect that the degradation potential with the both isolates (EB1 and G1) decreased detectable by proceeding degradation process due to decreasing pH.

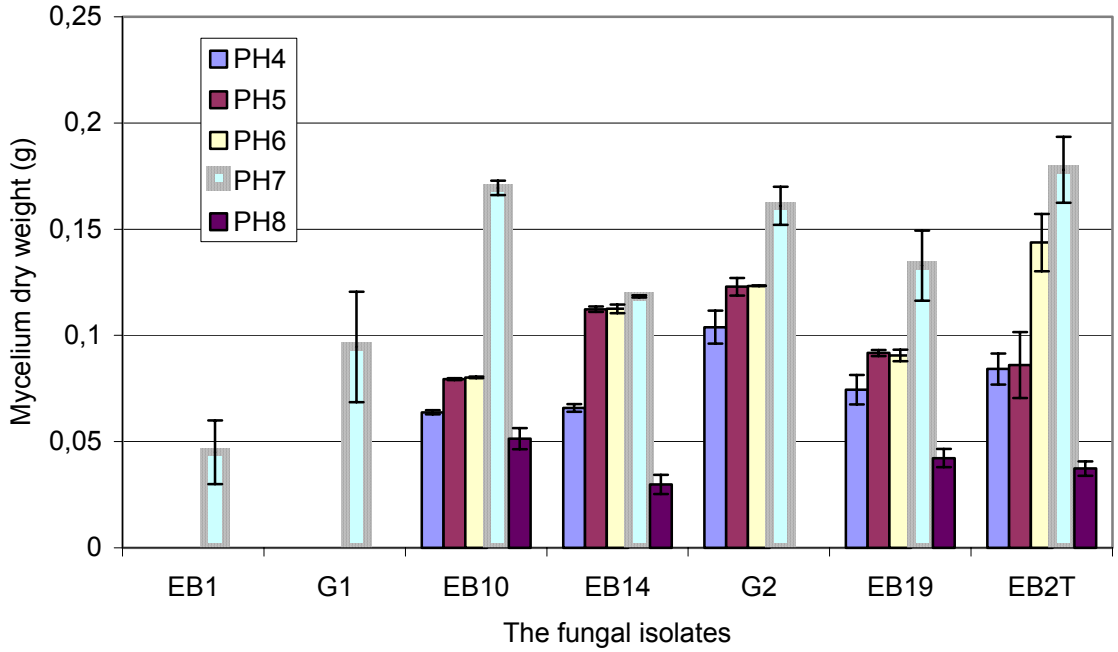


Fig. 3.7. Effect of pH on the mycelium dry weight for the selected fungal isolates
Medium: 30 ml MSV liquid -/ flask100 ml, Glucose: 15-20 g/L (20 g/l for EB1, G1, EB10, EB14, G2, EB19 and 15 g/l for EB2T) Temperature: 30 or 15°C, Incubation time: 1 week, Inoculum: 1disc/flask, n: 3 replicates

3.4.1.2. Optimal Temperature

In Fig 3.8 the effect of different temperatures on the growth of the fungal isolates on glucose is shown. A temperature of 30°C appears to be the optimum for the growth of EB1 and EB19, while EB10, EB14, G1, and G2 grew best at 25°C and EB2T at 15°C. Only one isolate (EB19) exhibited a growth at 40°C, while EB2T not grew even at 30°C. All fungal isolates exhibited a significant growth even at low temperatures (4°C).

The large variation of the fungal growth at different temperatures is normal, because many fungi causes diseases for plants and they are distributed in different environments at different temperature. Several plant pathogens and saprophytes can grow on cutin as the sole source of carbon. Suggesting that these organisms excrete a cutinase such as *Fusarium solani f. pisi*, *Colletotrichum capsici*, *Venturia inaequalis*. (Purdy and Kollattukudy 1973, 1975, Ettinger et al., 1978, Lin and Kolattukuddy 1980, Köller and Parker 1989 Fett et al., 1992 and 1994, Fan and Köller 1998, Cunha et al., 2003). Therefore this allows to use these isolates for the polyester degradation at these temperatures.

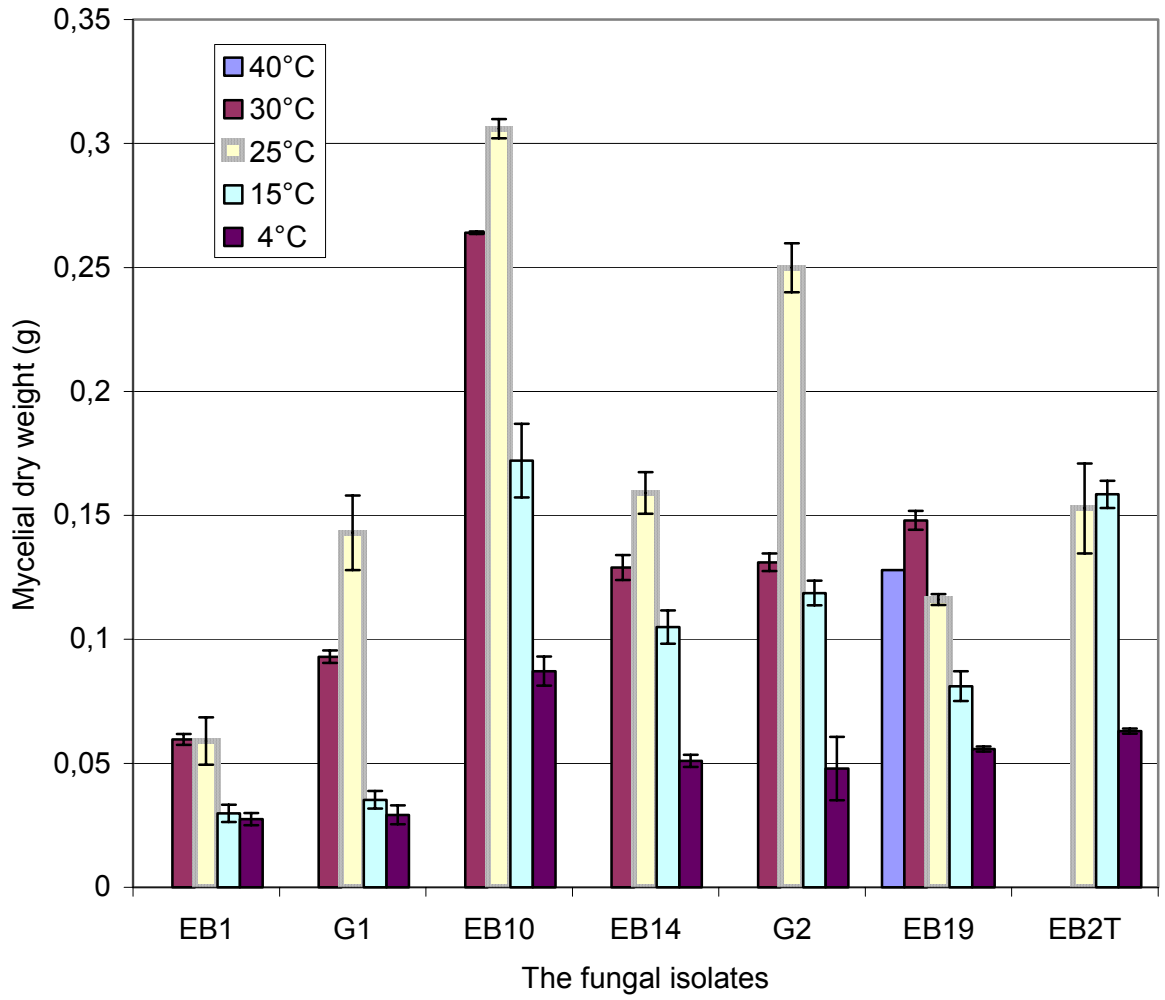


Fig. 3.8. Effect of temperature on the mycelium dry weight for the selected fungal isolates

Medium: 30 ml MSV-liquid/flask Inoculum: 1 disc /flask pH: 7

Glucose concentration: 15-20 g/l (20g/l for EB1, G1, EB10, EB14, G2, EB19 and 15 g/l for EB2T) Incubation time: 1 week

n: 3 replicates

3.4.2. Polyester degrading bacterial isolates

3.4.2.1. Optimal pH

The influence of pH on growth of the selected bacterial isolates is shown in Fig 3.9. Generally, the optimal pH was pH7 for all the isolates. The maximum intracellular protein was recorded at pH7. Most of the bacterial strains are known to prefer the neutral pH. As mentioned above for the fungal isolates that the variation of the growth at different pH leads to tolerance of the organisms to the pH changes during the degradation process. Therefore, it can be deduced from the results that the bacterial strains are sensitive to the pH changes during the degradation process.

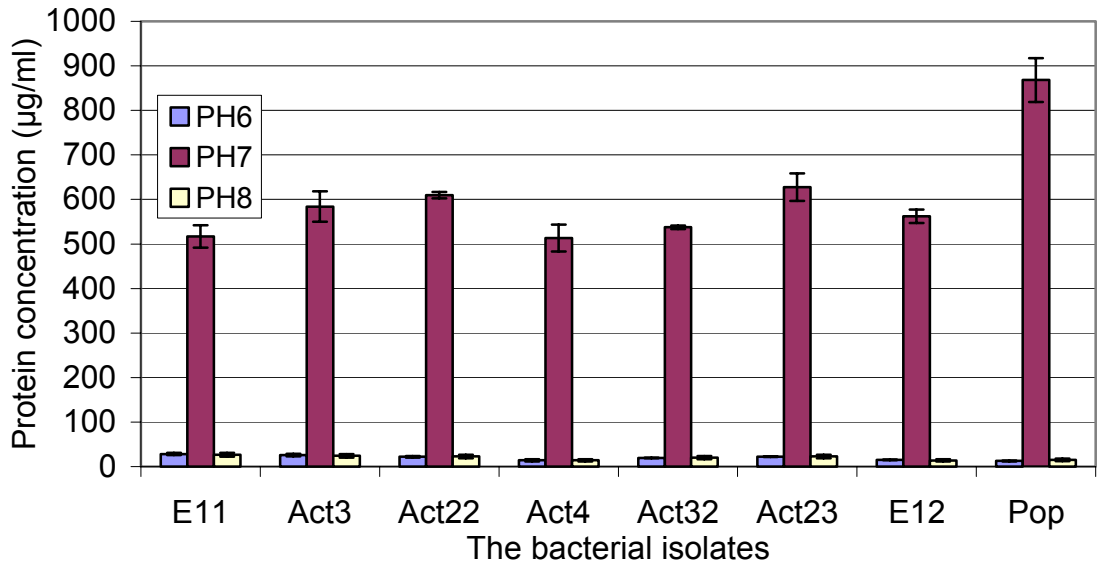


Fig. 3.9. Effect of pH on the growth for the selected bacterial isolates

Medium: 30 ml MSV-liquid/flask, Glucose concentration: 8 g/L Temperature: 30°C, Incubation time: 3 days for general bacteria/4 days for actinomycetes with shaking at 160 rpm for both groups. Inoculum: 1 ml (10^6 to10^7 for actinomycetes and bacteria, respectively), n : 3 replicates

3.4.2.2. Optimal temperature

In Fig 3.10 the effect of different temperatures on the growth of the bacterial isolates on glucose is shown. A temperature of 30°C appears to be the optimum for all isolates. All

bacterial isolates grew at 25°C but five of them grew at 40°C. Only three strains (Act4, E12 and Pop) did not grew even at 40°C.

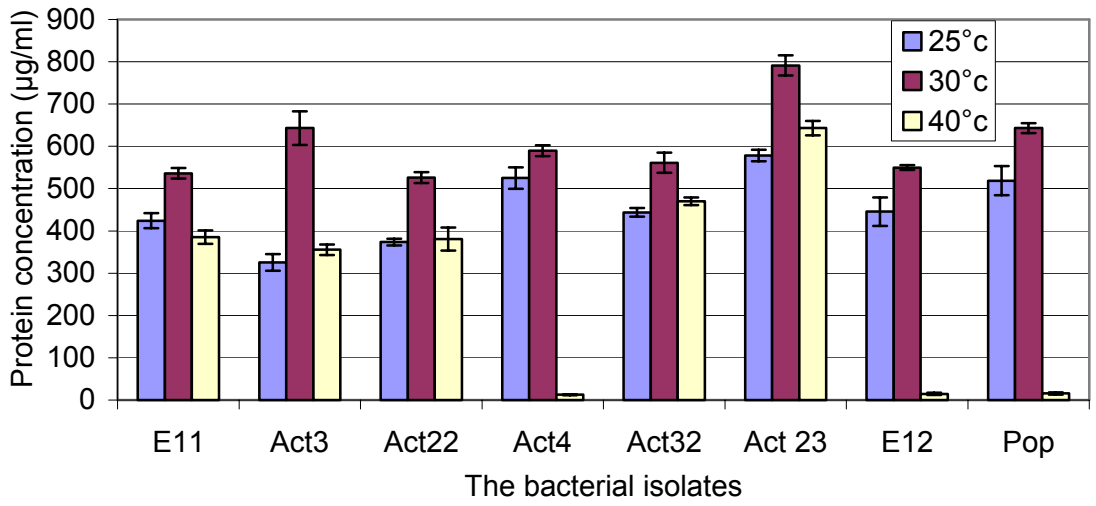


Fig.3.10. Effect of temperature on the growth for the selected bacterial isolates
Medium: MSV-liquid (30 ml MSV liquid/flask 100 ml), Glucose concentration: 8 g/L pH: 7, Temperature: 30°C Incubation time: 3 days for general bacteria/4 days for actinomycetes with shaking at 160 rpm., Inoculum: 1 ml/flask (10^6 to10^7 for actinomycetes and bacteria, respectively)

None of the polyester degrading bacterial isolates were able to grow at lower temperature such as 15°C and 4°C on complete agar medium (GYM medium for actinomycetes and LB medium for general bacteria).

Obviously, the range of physiologically acceptable conditions of the fungal isolates is more versatile in comparison with the bacterial isolates (actinomycetes and bacteria). It can be anticipated that the fungal isolates can degrade the tested polyester at these conditions. Therefore, the fungal isolates were used for further investigation with the aim of the determination of the biodegradation potential for the two commercialized polyesters (BTA 45:55 (Ecoflex) and PCL) at low temperatures (25, 15 and 4°C) (see chapter 3.7).

Two types of microorganisms are of particular interest in the biodegradation of natural and synthetic polymers; these are bacteria (general bacteria and actinomycetes) and fungi. Many species or types of microorganisms are found broadly in nature. These microorganisms play an outstandig role in the degrading of several natural polymers, like chitin, cellulose, starch, agar, and lignocelluloses (Crawford and Sutherland 1980; Tien and Kirk 1983; McCarthy and Cross 1984; McCarthy 1987; Kempf and Kutzner 1988).

3.5. Stability of the degradation character

After the isolation procedure the isolates were cultivated and preserved in the first phase of the work on complex media providing sufficient cell densities for culture maintenance. 12 isolates irreversibly lost their BTA-copolyester and PCL depolymerizing ability as a result of this preservation and repeated cultivation of the isolates on complex media.

Due to this instability of the BTA-copolyester and PCL degrading strains, an optimization of the culturing and preservation techniques was necessary. Instead of using complex media mineral salt vitamin agar media supplemented with the BTA-copolyester or PCL was applied as preservation technique for the degrading isolates. The instability found by the isolated strains may point to the involvement of a plasmid encoded character rather then a chromosomal one. Wiegand et al. (1999) observed the loss of the degradation ability by the isolated aerobic BAK 1095 (a biodegradable random copolymer of polyester and amide, developed by Bayer AG) when there was temporarily no selection pressure during the growth on BAK 1095 as the sole source of carbon. They explained this findings by an extrachromosomal codation of the degradation character. Abou- Zeid et al., (2001) reported also this phenomenon for polyester anaerobic degrading bacterial strains.

3.6. Identification and characterization of selected isolates

The stable fungal isolates EB1, EB10, EB14, EB19, EB2T and the strains E11, Act3, and Act23 as actinomycetes were taxonomically identified. The identification was performed by the DSMZ-Braunschweig, Germany.

3.6.1. The fungal isolates

This fungal isolates EB1, EB10, EB14, EB19 and EB2T were identified by DSMZ-Braunschweig, Germany according to colony and morphology characteristics as *Arthrobotrys amerospora, Fusarium solani, Acremonium strictum, Aspergillus fumigatus, Cladosporium herbarum*, respectively because they discriminate with these following characteristics with respect to colony and morphology measurements which are tabulated in Table 3.6. These isolates were designated with number and deposited in DSMZ as follow, *Arthrobotrys amerospora* DSM 15963*, Fusarium* solani DSM 15964*, Acremonium strictum* DSM 15965*, Aspergillus fumigatus* DSM 15966*, Cladosporium herbarum* DSM 15967. These isolates EB1, EB10, EB14, EB19 and EB2T was isolated previously by Schenck, Kendrik and Pramer, (marius) saccardo, Gams, (Pers.:Fr) Link as first authors, respectively.

Table 3.6. Colony and morphological characteristics of fungal strains EB1, EB10, EB14, EB19 and EB2T.

Fungal strains	Colony characteristics	Morphological characteristics
EB1	Colonies of EB1 are on malt extract agar at 25°C colorless, weakly matted aerial mycelium, the back of culture is weakly brownishly.	Conidiophores are colorless and thin-walled, for aerial mycelium or (particularly on corn flour agar) going out from the substrate and up to 250 µm long. Conidia formation is sympodial at short, cylindrical toothed and up to 10 conidia. The conidiophores are rarely proliferous and form from one to two conidia group. Aleurioconidia are colorless, unseptated and usually 2×14µm in size. This isolate produces chlamydospores. A special hyphae system for trapping the nematode was not observed. Strain EB1 was identified as ***Arthrobotrys amerospora*** schenck, Kendrick & Pramer
EB10	The growth rate on oat agar at 25°C is about 0.5 cm/day. The mycelium is colorless and inconspicuously to finely matted. Agar medium is slight brownish. No sclerotien is found in this strain. The sporodochien is cream to slightly orange. The isolate grew good at 37°C.	Microconidia are in slimy small head on very long a awlishy courteous phialids (up to 75 µm) in aerial mycelium. Microconidia are very variable; Usually they are ellipsoid to kidney-shaped; septated from one to two cell and up to 10 ×3-4 µm in size; Macroconidia are at shorter phialids, banana-shaped, nearly thick-walled, usually three septets, 30 × 4.5 µm in size, with no clear foot cell, point run dish and usually weakly curved. Chlamydospore are rarely, thick-walled, individually or in pairs and up to 10 µm in diameter. Strain EB10 was identified as ***Fusarium solani*** (marius) saccardo
EB14	The cultures exhibit on oat agar at 25°C a diameter of 18 mm after 6 days. At first the culture is colorless. The aerial mycelium is in cluster and later it is slightly orange. The back of culture for this strain is palely.	The mycelium is very tenderly and the hyphae diameter is about 1-1.5 µm. No conidiophore is found in this strain. Phialids are simply, awlishy, no chromphil, laterally undifferentiated hyphae and up to 40 µm long. Conidia are cylindrical/ellipsoidal 5 × 1 µm and in slimy small head. This strain did not produce chlamydospores. Strain EB14 was identified as ***Acremonium strictum*** Gams

Table 3.6.

Fungal strains	Colony characteristics	Morphological characteristics
EB19	The isolate EB19 is quickly growth on malt extract agar at 25°C and the growth rate is 6 mm/day. The mycelium is inconspicuously and colorless. Conidia mass are darkly blue-green and the back of culture for this strain is palely. It grew very good at 45°C.	Conidiophore is straight 200-300 µm long, up to 7 µm in diameter, smoothly and thin-walled; at the end it was extend gradually to a vesicle of 18-20 µm in diameter. No metulae is found in this strain; Phialids are aligned parallel to the conidiophore and they are about ¾ the vesicle surface area, 6-7 µm long and bottle-shaped. Conidia are nearly ball, 2.5-3 µm in diameter and smoothly. Strain EB19 was identified as ***Aspergillus fumigatus*** Fresenius
EB2T	The isolate shows spreading growth on malt extract agar at 25°C and the growth rate is about 2 mm /day. Colony is flat, velvety, olive-green and it back is black-green. The isolate did not grow at 37°C.	Conidiophore is brownishly, smooth-walled and proliferating. Conidia are acropetal sprout-end, federations easily disintegrating and clear scars with separation of the conidian. Terminal conidia are ellipsoidal, 6-12× 4-6 µm and wart. Strain EB2T was identified as ***Cladosporium herbarum*** (Pers .:Fr.) Link

These different genera from fungal strains are heterotrophic organisms living as saprophytes or parasites in soil. These strains were used in different purposes in the nature by many authors as follow:

Arthrobotrys is a soil-living fungus that can trap and infect nematodes using special forming a three –dimensional net. Species of this fungi produce high levels of a saline-soluble and low –molecular-mass lectins (Rosén et al., 1992). No literature report has been found on the biodegradation of the polyesters by this genus.

Fusarium solani is found in the soil everywhere and can degrade glycogen, inuline, starch, and cellulose (Domsch et al., 1980). *Fusarium solani* was isolated from the soil as the microbe that degrades PCL (Benedict et al., 1983). Cutinase from the phytopathogenic fungus *Fusarium solani f.sp. pisi* is an example of a small carboxylic ester hydrolase that bridges functional properties between lipases and esterases. Cutinases are named after their ability to degrade cutin polymers (Kolattukudy 1984). *Fusarium solani* was isolated based on their ability to utilize a colloidal polyester PU (Impranil DLN^{TM}) as the sole carbon and energy source (Crabbe et al., 1994). Polycaprolactone (PCL) is degraded by *Fusarium solani f. sp. pisi* and Fusarium polycaprolactone depolymerase is cutinase (Murphy et al.,1996). It is known to be effective in degrading PHB and Mater-Bi (MB) (Kim et al., 2000).

Species of Acremonium are very common soil fungi and are known to produce various hydrolytic enzymes (Domsch et al., 1980). It is known that lipase was produced by *Acremonium strictum* (Okeke and Okolo 1990). Roßhaupten and Molitoris (1996) reported earlier that *Acremonium rutilum* can degrade BTA 40:60 a copolyester.

Aspergillus fumigatus is found all over the world and it grow on glucose, maltose, sucrose, lactose, cellulose, inuline, starch, casein, peptone, urea, or cyanamide (Domsch et al., 1980). It is known to be effective in degrading plasticized PVC and DDT (Domsch et al., 1980). *A. fumigatus* has also been reported to produce such enzymes as amylase, lipase, and phosphatase, and its purified PHB depolymerase is known to have a very high hydrolysis activity for aliphatic polyesters (Scherer 1999). It is known to be effective in degrading of three commercial biodegradable plastics, poly(3-hydroxbutyrate) (PHB), Sky-Green® (SG), a biodegradable aliphatic polyester made of succinic acid, adipic acid, butandiol and ethylene glycol, and Mater-Bi® (MB) (Kim et al 2000). *A. fumigatus* has also been reported to produce such enzymes as β-xylosidase (Lenartovicz et al., 2002).

Cladosporium sp. was isolated based on it ability to utilize a colloidal polyester PU (Polyurethane) (Impranil DLN^{TM}) as the sole carbon and energy source (Crabbe et al., 1994). *Cladosporium herbarum* was effective in the biodegradation of anthracene (Krivobok et al., 1998).

3.6.2. Bacterial isolates (E11, Act3 and Act23)

Actinomycetes isolates were identified with respect to morphology, physiology as well as molecular biology (phylogenetic) by using analysis of 16S rDNA. The identification was performed in DSMZ-Braunschweig, Germany. The results shown in Table 3.6 summarizes the morphology, physiology as well as 16S rDNA for strains E11, Act3 and Act23.

Table 3.7. Morphological, physiology and 16S rDNA for stains E11, Act3, Act23.

	Strain	Color aerial mycelium	Color substrate mycelium	Soluble pigment	Spore chain morphology	Melanin formation	Cell wall DAP
Morphological characteristics	E11	pink	orange-brown	no	pair	no	DL
	Act3	pink	orange-brown	no	pair	no	DL
	Act23	gray	dark-brown	no	spiral	no	LL
Physiological characteristics	E11 / Act3	According to the results of utilization the C-source spectrum or other physiological comparison date the both strains are not classified to any species for *Microbispora*. By using "Rhodococcen substrate" the both strains were classified (69 and 74%) to *R. fascians.*					
	Act23	According to the results of utilization the C-source which were showed only little correlation (97%) with the cluster *S. rochei* of the physiology date bank. With this method, it can not be help to classify for these isolate to *S. thermocarboxydus* (Kämpfer et al. 1991).					
Chemotaxonomic by analysis fatty acids	E11 / Act3	With the fatty acids pattern for two isolates, they were classified to *Microbispora rosea subsp. rosea*. The fatty acid pattern of the strains contained mainly iso-and anteiso-branched fatty acids as 10-methyl branched, and 2-hydroxy fatty acids					
	Act23	The fatty acid pattern which contained iso-and anteiso-branched, but no 10-methyl-branched and also 2-hydroxy fatty acid were found in *Streptomyces* . With the fatty acid, this strain was classified to *S. rochei*.					
Genetic identification by using 16S rDNA	E11 / Act3	According to the 16S rDNA analysis the two selected strains E11 and Act3 show the similarity of 99.5% to *Microbispora rosea subsp. rosae* (IFO 14044^{T}), and 99.8 % to reference strain DSMZ 46159. The base sequence (1-500 nt) was identical for both isolates. Both strains were identified as *Microbispora rosea subsp. rosea*.					
	Act23	According to the 16S rDNA analysis the selected strain Act23 show the similarity of 100% to *Streptomyces thermocarboxydus*.					

These isolates were designated with number and deposited in DSMZ as follow, *Microbispora rosea subsp. Rosea* DSM 44767 (E11) and DSM 44768 (Act3) and *Streptomyces thermocarboxydus* DSM 41841.

Microbispora rosea subsp. rosae produce bronze-violet crystals of iodinin (1,6- phnenazine-5,10-dioxide) by cultivation on medium containing starch.

It was observed *Streptomyces thermocarboxydus* produce braun pigments during the growth on starch mineral salt medium.

Actinomycetes are widely distributed in natural environments, such as soils and composts where they make an important contribution to nutrient recycling and humification. They are therefore a potentially useful source of plant biomass-degrading enzymes, and activity against the major components (lignin, hemicellulose and cellulose) has been identified in many strains (McCarthy 1987).
Straw-saccharifying activity was further characterized in selected strains comprising the genera *Streptomyces* and *Microbispora* (Ball and McCarthy 1988).

3.7. Use of the polyesters degrading fungal isolates for degradation tests under natural conditions (25, 15 and 4°C).

The 5 fungal strains were isolated from mature compost and garden soil which were identified as *Arthrobotrys amerospora* (EB1), *Fusarium solani* (EB10), *Acremonium strictum* (EB14), *Aspergillus fumigatus* (EB19), *Cladosporium herbarum* (EB2T) as well as two strains from *Gliocladium roseum* (G1 and G2) exhibited degradation potential against BTA-copolyester (BTA 45:55 (Ecoflex) and BTA 40:60), PCL and SP46 as synthetic polyesters at 30°C. Data, which are shown in 3.4.1.2 demonstrate that most of these strains can grow from 4°C to 30°C on mineral salt vitamin liquid with glucose. Degradation experiments with polyesters were up to this stage only performed at 30 °C. The question arises now if the isolates can degrade the polyesters at lower temperatures (25°C, 15°C and 4°C) which better represent the situation often present under natural conditions (e.g. degradation in soil).

3.7.1. Evaluation of the biodegradability of BTA 45:55 (Ecoflex) and PCL with the fungal strains.

The fungal isolates were tested with dispersal BTA 45:55 (Ecoflex) and PCL in mineral salt vitamin agar media. Clear zone formation was used as a sign for the biodegradation of the polyesters as a preliminary test at the different temperatures. The results in Table 3.8 show that most of the isolates degrade both polymers by forming clear zone even at 25°C, 15°C and 4°C. Only one fungal isolate (EB19) did not form any clear zones at low temperature (15 and 4°C). This indicates that there is no correlation between the growth and the degradation process at low temperatures (15 and 4°C) since the strain EB 19 was growing at low temperatures on mineral salt medium with glucose. It was of interest now to determine the degradation potential more quantitatively by the determination of the degradation rate by weight loss measurements with polymer films on agar plates.

Table 3.8. Clear zone formation by the fungal isolates on MSV-BTA 45:55 (Ecoflex) or PCL at different temperature after 1 week.

Medium: Mineral salt vitamin agar, Polymer: BTA 45:55 (Ecoflex) (0.1%), Inoculum: 1 disc from complex medium (5 mm in Diameter bearing the fungal mycelium), Incubation time: 1week.

Fungal isolates	BTA 45:55 (Ecoflex)			PCL		
	25°C	15°C	4°C	25°C	15°C	4°C
EB1	+	+	+	+	+	+
EB10	+	+	+	+	+	+
EB14	+	+	+	+	+	+
EB19	+	-	-	+	-	-
G1	+	+	+	+	+	+
G2	+	+	+	+	+	+
EB2T	+	+	+	+	+	+

+ formation of clear zones

- no clear zones

The degradation potential of the selected fungal strains was characterized as weight loss of polyester films on mineral salt media agar plates after different incubation times depending on the degradation temperature (6 weeks for 25°C and 15°C and 32 weeks for 4°C)

The results in Fig. 3.11 and Table 3.9 show the difference in the degradation rate of the fungal isolates at different temperatures (25, 15 and 4°C).

Generally, BTA 45:55 (Ecoflex) films are degraded most rapidly with most isolates at 25°C, followed by the degradation rate at 15°C and at 4°C. The isolates EB1 and G1 degraded BTA 45:55 (Ecoflex) films completely at 25°C after 6 weeks. These isolates exhibit a remarkable degradation potential which is about 3-10 fold higher than the other isolates at 25°C. It was surprising that BTA 45:55 films as partially aromatic copolyester were degraded significantly also at lower temperature such as 15 and 4°C. At 15°C the strains EB1 and EB2T were most active and degraded the polymer films about 1-2 fold faster than the other fungal isolates. At 4°C the strains EB10, EB14 and EB2T degraded BTA 45:55 (Ecoflex) about 5-10 fold higher than the fungal strains. In conclusion it can be stated that the strain

EB1 plays an outstanding role in the degradation of BTA 45:55 (Ecoflex) films at 25 and 15°C, while at 4°C EB10 exhibited the highest degradation potential.

Obviously, the general trend of the biodegradability of the polyester with the fungal strains with regard to the temperature depends on the particular strain. The degradation rates of the strains at the different temperatures are the following:

25°C: EB1 ~ G1 > EB14> EB19> G2> EB10> EB2T

15°C: EB1 > EB2T> EB14> G1> G2> EB10

4°C: EB10> EB14> EB2T> G2> EB1~ G1

The experimental results were compared with those obtained from the noninoculated films (control), which showed no weight loss under the test conditions applied. Fig. 3.12. shows the growth of EB1 on Ecoflex films at 25°C, where the films were degraded completely. Fig.3.13. shows examples of growth for the fungal strains on BTA 45:55 (Ecoflex) films at 4°C.

Table 3.9. Degradation rate for BTA 45:55 (Ecoflex) films [mg/week.cm²] with the fungal strains at different temperatures.

Medium: mineral salt vitamin agar, polymer: BTA 45:55 (Ecoflex) films, Diameter: 25 mm Surface area: 4.91cm², films weight: 28.3 – 33.23 mg, thick of the films: 60 µm, temperature: 25, 15, and 4°C, Incubation time: 6 weeks at 25 and 15°C, and 32 weeks at 4°C, Inoculum: 1 disc (5 mm in diameter) from complete medium bearing the fungal mycelium.

Fungal strains	Degradation rate [mg/week.cm²]		
	25°C	15°C	4°C
Arthrobotrys amerospora (EB1)	>1.14	0.41	0.021
Fusarium solani (EB10)	0.255	0.22	0.16
Acremonium strictum (EB14)	0.359	0.321	0.112
Aspergillus fumigatus (EB19)	0.286	-	-
Cladosporium herbarum (EB2T)	0.11	0.357	0.1
Gliocladium roseum (G1)	> 1.11	0.298	0.02
Gliocladium roseum (G2)	0.277	0.26	0.03

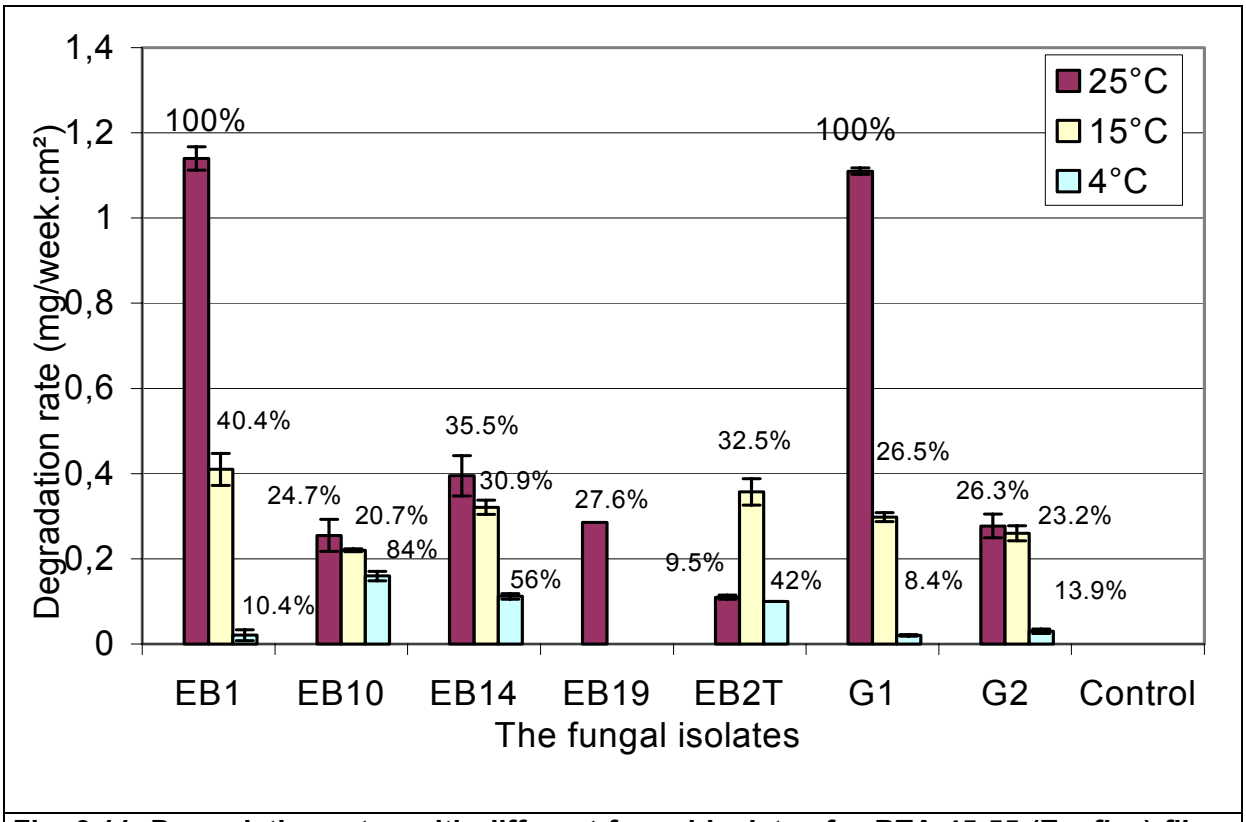
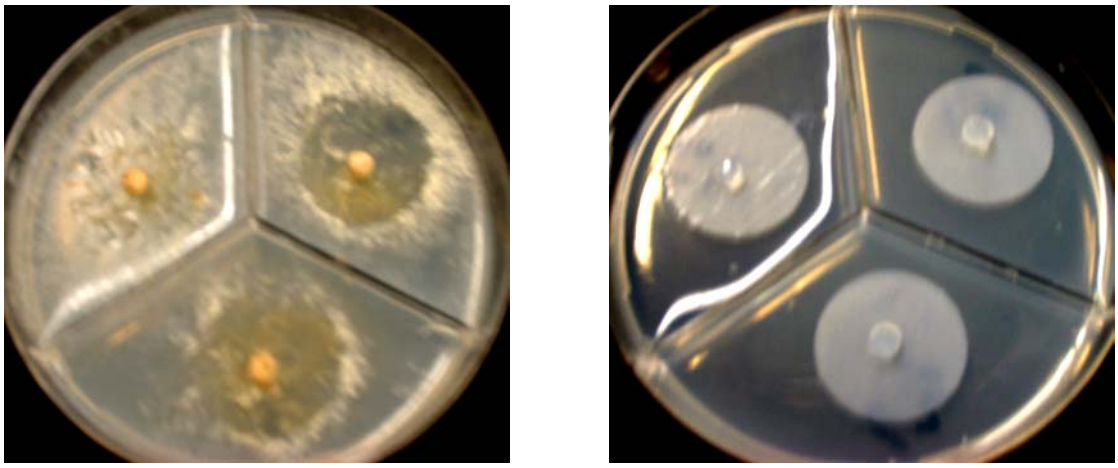


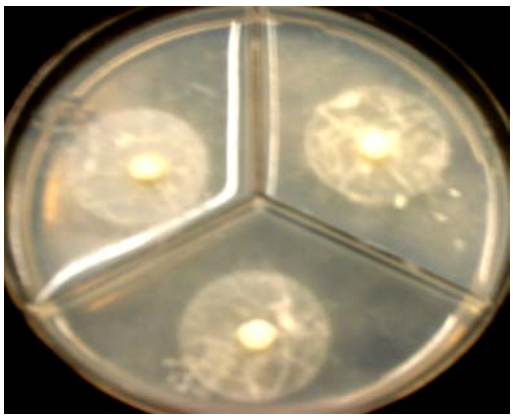
Fig. 3.11. Degradation rates with different fungal isolates for BTA 45:55 (Ecoflex) films on MSV-agar at different temperature.

Medium: mineral salt vitamin agar, polymer: BTA 45:55 (Ecoflex) films, Diameter: 25 mm Surface area: 4.91cm², films weight: 28.3 – 33.23 mg , thick of the films: 60 µm , temperature: 25, 15, and 4°C, Incubation time: 6 weeks at 25 and 15°C, and 32 weeks at 4°C, Inoculum: 1 disc (5mm in diameter) from complete medium bearing the fungal growth.

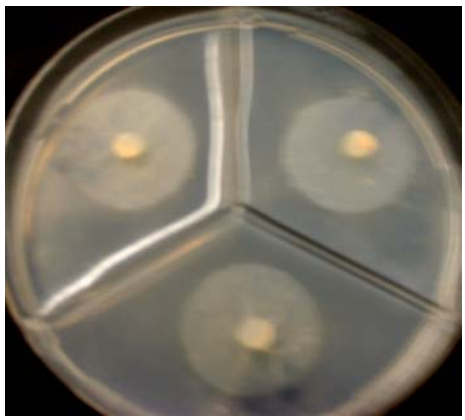


with *Arthrobotrys amerospora* (EB1) **Control BTA 45:55 (Ecoflex) films**

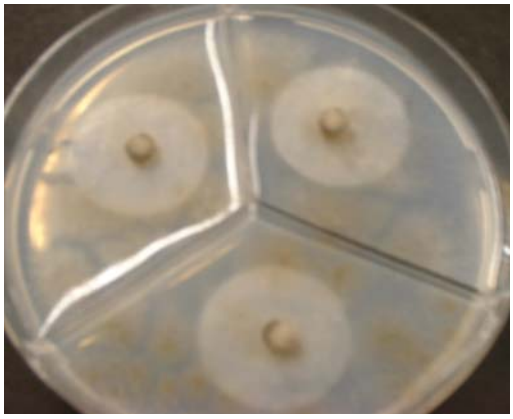
Fig. 3.12. *Arthrobotrys amerospora* (EB1) on BTA 45:55 (Ecoflex) films at 25°C after 6 weeks(the films were degraded completely). Control (films not inoculated with EB1).



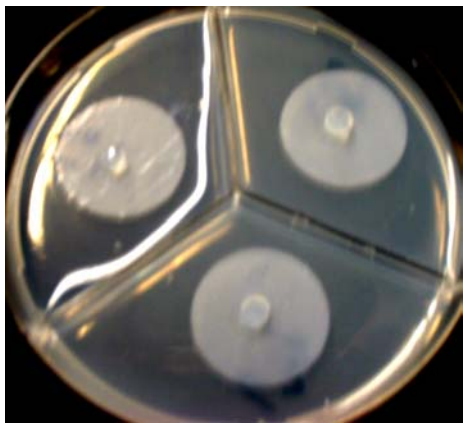
with *Fusarium solani* (EB10)



with *Acremonium strictum* (EB14)



with *Cladosporium herbarum* (EB2T)



Control BTA 45:55 (Ecoflex) films

Fig.3.13. Growth of the fungal strains on BTA 45:55 (Ecoflex) films on MSV-agar at 4°C. Control of BTA 45:55 (Ecoflex) films (not inoculated with the fungal strains)

The degradation potential of the fungal isolates at different temperatures were additionally characterized using the aliphatic polyester PCL, which exhibits a much lower melting temperature (approx. 60°C) than the BTA-copolyester. The results are shown in Fig. 3.14 and Table 3.10.

As observed for the BTA copolyester films PCL films were generally degraded faster with most isolates at 25°C and the degradation rate was decreased at lower temperatures. The general trend of the degradation potential was for these strains is as follow:

25°C EB10> EB14> G1> EB19> EB1> G2> EB2T

15°C EB10> G1> EB14> EB2T> EB1> G2

4°C EB10 ~ EB14> EB2T> G1> G2> EB1

Obviously, the EB10 isolate prevailed over the other isolates at 25 and 15°C. At 4°C EB10, EB14 and EB2T exhibit a degradation potential about which is about 3-30 folds more than the other isolates. The results were compared with those obtained with noninoculated films (noninoculated controls) which were incubated in the respective media. The noninoculated controls showed no weight loss under the test conditions. Fig. 3.15 shows examples for the growth of degrading fungal strains on PCL films at 4°C on MSV-agar.

Table 3.10. The degradation rate of PCL films with fungal strains at different temperatures.

Medium: mineral salt vitamin agar, polymer: PCL films, Diameter: 25 mm, surface area: 4.91cm², films weight: 39.53 – 42.9 mg, thick of the films: 75 µm, temperature: 25, 15, and 4°C, Incubation time: 6 at 25 and 15°C, and 32 weeks at 4°C, Inoculum: 1 disc (5mm in diameter) from complete medium bearing the fungal growth.

Fungal strains	**Degradation rate [mg/week.cm²]**		
	25°C	15°C	4°C
Arthrobotrys amerospora (EB1)	0.592	0.584	0.001
Fusarium solani (EB10)	>1.4	1.3	0.3
Acremonium strictum (EB14)	1.34	1.13	0.3
Aspergillus fumigatus (EB19)	1.02	-	-
Cladosporium herbarum (EB2T)	0.103	0.898	0.264
Gliocladium roseum (G1)	1.24	1.2	0.14
Gliocladium roseum (G2)	0.566	0.331	0.005

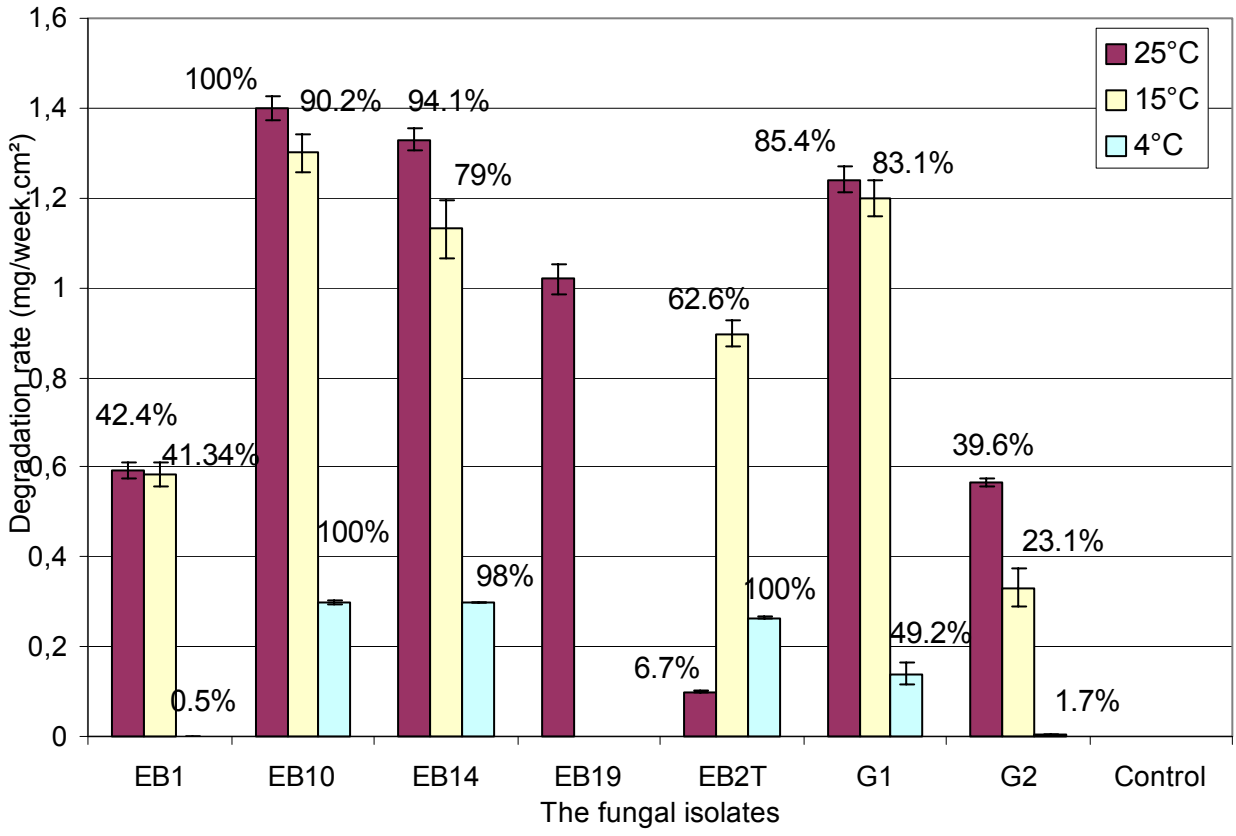


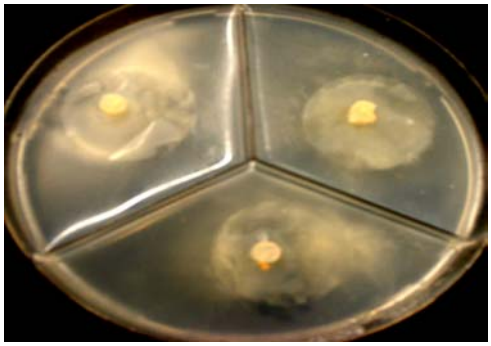
Fig. 3.14. Estimation of degradation rate [mg/week.cm²] with different fungal isolates for PCL films on MSV-agar at different temperature.

Medium: mineral salt vitamin agar polymer: PCL films, Diameter: 25 mm

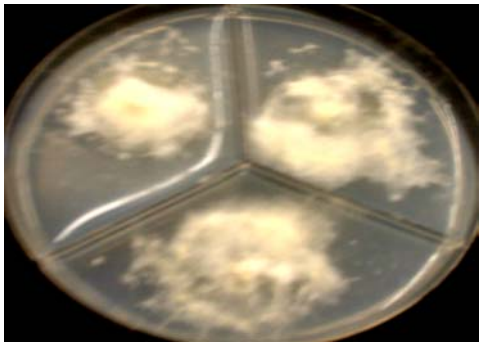
surface area: 4.91cm², films weight: 39.53 – 42.9 mg, thick of the films: 75 µm ,

temperature: 25, 15, and 4°C,

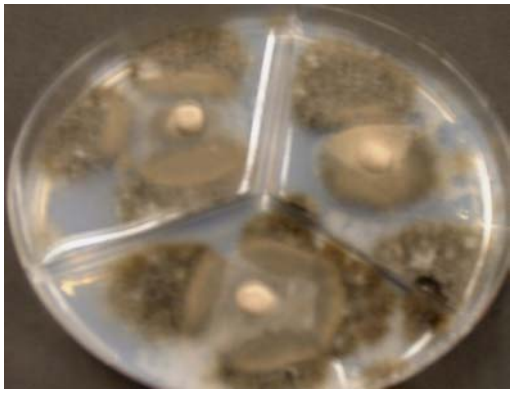
Incubation time: 6 weeks at 25 °C and 15°C, and 32 weeks at 4°C, Inoculum: 1 disc (5mm in diameter) from complete medium bearing the fungal growth



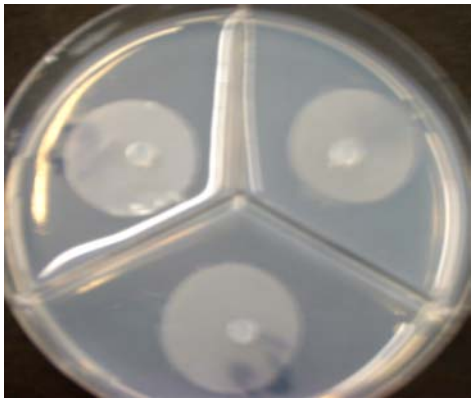
with *Acremonium strictum* (EB14)



with *Fusarium solani* (EB10)



with *Cladosporium herbarum* (EB2T)



Control PCL films (noninoculated)

Fig. 3.15. Growth of the fungal isolates on PCL films on MSV-agar at 4°C. Control of PCL films (noninoculated with the fungal strains)

From the described results it can be concluded that the fungal strains exhibited remarkable degradation for the two polyesters at different temperature. Therefore, It can be stated that the fungi play an important a role in the biodegradation of polyester films at different temperatures.

PCL films were degraded faster than BTA 45:55 with most isolates at different temperature. Marten (2000) postulated for instance that the reduced flexibility of the copolyester chains, which correlates with the relative high melting points of the copoylesters, hinders the polymer chains to adjust into the active site of the hydrolyzing enzymes.

However, one of the isolates (*Arthrobotrys amerospora* EB1) exhibits a higher degradation potential against BTA 45:55 (Ecoflex) films than against PCL films at 25 and 4°C, but degrades PCL films faster than BTA 45:55 (Ecoflex) at 15°C. Also one *Gliocladium roseum* strain can degrade BTA 45:55 faster than PCL at 4°C.

The isolate EB19 could degrade none of the synthetic polyesters at low temperatures (15 and 4°C) despite it grew at the same conditions in mineral salts vitamin liquid medium supplemented with glucose as a carbon source. This is probably due to the fact that this strain does not secrete the hydrolytic enzyme at low temperatures. This clearly indicates that there is obviously no correlation between the growth of the fungi and the biodegradation process.

The fungal strains EB10, EB14 and EB2T play an outstanding role in the biodegradation for BTA 45:55 (Ecoflex) and PCL films at 4°C. From the 7 fungal strains, three strains (EB10, EB14 and EB2T) are known as phytopathogenic. Several plant pathogens are widely distributed in different environments and can grow on cutin as a sole carbon source, suggesting that such organisms possibly secrete a cutin hydrolyzing enzyme (Purdy and Kollattukudy 1973, 1975, Ettinger et al., 1978, Lin and Kolattukuddy 1980, Köller and Parker 1989 Fett et al., 1992 and 1994, Fan and Köller 1998, Cunha et al., 2003). Many authors used phytopathogenic fungi to degrade synthetic polymers such as polycaprolactone (PCL), where the isolates secrete cutinase that degrades cutin in the structural polymer of the plant cuticle (Oda et al., 1995, Murphy et al., 1996, Nishida et al., 1999).

As mentioned above the fungal strains were used in degradation of the two polyesters at 30°C (chapter 3.2.2.1). Therefore it can compare between the degradation rate for the two polyesters at 30°C and at low temperatures with these strains. Table 3.11 summarizes the difference of the degradation rate between BTA 45:55 (Ecoflex) and PCL at 30, 25, 15 and 4°C. Generally, it was observed in most cases that by increasing the temperature, the degradation potential was significantly increased and the incubation time decreased. PCL films were degraded faster at 30°C than at 25, 15 and 4°C with strains EB1, EB10, EB14, G1 and G2. Also BTA 45:55 (Ecoflex) films were degraded faster with increasing the temperature from 4 to 30°C with EB10, EB14 and EB19. On the other hand BTA 45:55 (Ecoflex) films were degraded faster at 25°C than 30, 15 and 4°C with strains EB1, G1 and G2; also PCL films were degraded faster at 25°C faster than at 30°C with strain EB19. The two polyesters were degraded by strain EB2T at 15°C faster than at 25 and 4°C. This clearly indicates that the degradation rate depend on the strain, incubation temperature and the type of polyester.

Table 3.11. The difference of the degradation rate [mg/week.cm²] between BTA 45:55 (Ecoflex) and PCL films with fungal strains at 30, 25, 15 and 4°C.

Medium: mineral salt vitamin agar, polymer: PCL films, Diameter: 25 mm, surface area: 4.91cm², Initial weight of the films: PCL: 39.53 – 42.9 mg, BTA 45:55 (Ecoflex): 26.83 – 33.23 mg, thickness of films: PCL: 75 µm, BTA 45:55 (Ecoflex): 60 µm, temperature: 30, 25, 15, and 4°C, Incubation time: 4 weeks at 30°C, 6 weeks at 25 and 15°C, and 32 weeks at 4°C, n = 3 films per test, Inoculum: 1 disc (5mm in diameter) from complete medium bearing the fungal growth.

Fungal strains	Degradation rate [mg/week.cm²]							
	30°C		25°C		15°C		4°C	
	BTA45:55 (Ecoflex)	PCL	BTA45:55 (Ecoflex)	PCL	BTA45:55 (Ecoflex)	PCL	BTA45:55 (Ecoflex)	PCL
Arthrobotrys amerospora (EB1)	0.62	1.57	1.14	0.592	0.41	0.584	0.021	0.001
Fusarium solani (EB10)	0.263	2.1	0.255	1.4	0.22	1.3	0.16	0.3
Acremonium strictum (EB14)	0.51	2.1	0.395	1.34	0.321	1.13	0.112	0.3
Aspergillus fumigatus (EB19)	0.332	0.9	0.286	1.02	-*	-*	-*	-*
Cladosporium herbarum (EB2T)	-**	-**	0.11	0.103	0.357	0.898	0.1	0.264
Gliocladium roseum (G1)	0.42	2.1	1.11	1.24	0.298	1.2	0.02	0.14
Gliocladium roseum (G2)	0.204	0.8	0.277	0.566	0.26	0.331	0.03	0.005

***EB19 does not form clear zone agar plates with these polyesters at these temperature.

**EB2T not grow at 30°C.

Kleeberg et al., (1998) reported that BTA 40:60 was degraded very fast with thermophilic actinomycetes strains such as *Thermomonspora fusca*. By comparison between the degradation rate under thermophilic conditions with *T. fusca* and mesophilic conditions in the present work, the degradation rate under thermophilic conditions was about 17.5 fold than under mesophilic conditions. The biodegradability enhancement under thermophilic conditions can be explained by the involvement of different organisms and an increase of the flexibility of the polyester chains with increasing incubation temperature.

Abou-Zeid et al., (2001) demonstrated that PCL films were depolymerized under anaerobic conditions by strain *Clostridium acetobutylicum* at 37°C. By comparison between the degradation rate for PCL films under anaerobic conditions with *Clostridium acetobutylicum* and the degradation rate with *Fusarium solani* and *Acremonium strictum* at range from temperature 4-30°C in the present work, the degradation rate was with *Fusarium solani* and *Acremonium strictum* at range from temperature 4-30°C about 10-73 fold higher than under anaerobic conditions.

3.8. Effect of the surface area on the degradation of BTA 45:55 (Ecoflex) by *Arthrobotrys amerospora* (EB1)

The strain EB1, identified as *Arthrobotrys amerospora* exhibited the highest degradation potential against BTA 45:55 (Ecoflex) films in comparison with the other isolates in the present work. With this model strain the question should be examined in which extent the surface area of the polyesters have an influence on the degradation. Two forms of BTA 45:55 (Ecoflex), powder and nanoparticles instead of films were used to investigate the effect of the surface area on BTA 45:55 (Ecoflex) degradation by strain *Arthrobotrys amerospora* (EB1) in liquid mineral salt vitamin medium.

3.8.1. Degradation of BTA 45:55 (Ecoflex) powder (0.1- 0.2 mm Ø) at 30°C by *Arthrobotrys amerospora* (EB1)

Conidia suspension (10^5 CFU/ml) for strain *Arthrobotrys amerospora* EB1 were grown in mineral salt liquid media (100 ml) containing BTA 45:55 powder (100 mg) (surface area A= 0.00307 m²/g) at 30°C and 160 rpm for different incubation times. The results illustrates the time course of degradation, which was quantified by measuring the degradation rate [mg/week. m²] of the polyester powder.

The degradation rate for BTA 45:55 (Ecoflex) powder was 5×10^3, 2.7×10^3, and 2.1×10^3 mg/week.m² after 4, 8, and 12 weeks, where the percentage of degradation was 62.6, 65 and 76.8 after 4, 8 and 12 weeks, respectively. It was observed from the results that the degradation rate for BTA 45:55 (Ecoflex) powder decreased after 8 - 12 weeks when

compared with the initial phase (4 weeks). This behavior can probably be attributed to an accumulation of an intermediates in the surrounding milieu or may be due to binding of inactive biomass to polyester surface and then this hinders attack of the polyester by the enzyme .

The same result was obtained by this strain after 12 week incubation under the same conditions, but in larger test set up (5 ml conidia suspension/flask (1L) containing 500 ml medium with 500 mg from BTA 45:55 (Ecoflex)). The degradation rate was 2.2 × 10^3 mg/week.m² after 12 weeks with 80.5 % degradation. The non-inoculated controls after 4 and 8 weeks showed 5 - 9 mg due to an experimental error in weight of the sample but the test set up running for 12 weeks exhibited degradation rate a 1.3 ×10^3 mg/week.m² of the polymer powder (49.4 % degradation). It was suspected that this degradation was because of a contamination with a fungal contaminant.

3.8.2. Degradation of BTA 45:55 (Ecoflex) nanoparticles (220 nm) at room temperature by *Arthrobotrys amerospora* (EB1)

16 discs (5 mm in diameter) from a agar plate with complex medium (potato dextrose agar) bearing the fungal mycelium were inoculated in mineral salt vitamin liquid medium containing BTA 45:55 (Ecoflex) nanoparticles (0.1% w/v) (surface area A= 20.98 m²/g). After 1 week of cultivation the degradation rate of BTA 45:55 (Ecoflex) nanoparticles was 30 mg/week.m² (62.5 % degradation). A non -inoculated test showed that 1.5 ± 7 mg were found again due to an experimental error.

By comparison between the degradation rate of BTA 45:55 (Ecoflex) powder and nanoparticles, the degradation rate was much higher by using the powder than the nanoparticles and this was not expected from the previous literature where the authors (Pranamuda el al., 1995) reported that the nanoparticles with large surface are degraded faster than the other form from polyester such powder or films which they discriminate with small surface area. The value of degradation rate with powder was 70 -166 fold higher than the degradation rate with nanoparticles. This is contradictive to previous literature which reported the degradation rate increased by increasing in the surface area (Abou-Zeid et al., 2001). Probably this may be due to the reason, of BTA 45:55 (Ecoflex) nanoparticles were aggregated after the cultivation and they became bigger particles sizes and small surface area and this not allow to attack all surface area with the enzyme; the incubation temperature is different between the both cases where it was at 30°C with powder experiments but it was

at room temperature with nanoparticles experiments; the difference of the type of inoculm, where it was used spore suspension with powder experiment but it was used discs from the complex medium (Potato Dextrose agar) which beard the fungal mycelium and existence this discs in the medium as complex medium may be inhibited the enzyme production.

By comparison between the degradation rate for either powder and nanoparticles and the degradation rate for films for BTA 45:55 (Ecoflex) with the same strain on agar plates, the degradation rate of films was much slower than the either forms (powder and nanoparticles) due to their larger particles and smaller surface area.

3.9. Characterization of the PCL-degradation enzyme system of strain E11

The strain E11 as actinomycetes was used in the following part despite the fungal strains exhibited degradation potential towards the tested polymers more than the actinomycetes on agar plates due to the reasons, that the fungi do not homogenously grow in liquid medium and it was observed that during growth of the fungi on dispersal polyesters in agar medium, the clear zone formation coincide with mycelium growth probably due to cell wall bound enzyme and it is very difficult to obtain on the enzyme from the cells when the fungi grew on agar plates.

PCL was used as substrate for strain E11 instead of BTA copolyester because PCL was degraded faster more than BTA copolyester by this strain. Gouda et al. (2002) found that some factors influence on production of BTA hydrolase which was produced by *Thermomnspora fusca*. The authors demonstrated that BTA hydrolase was not produced detectably in the supernatant of the culture due to its adsorption on the undegraded BTA polymer surface.

Having determined up to now the main factors influencing the PCL degradation process with strain E11, it is aimed in the following section to gain basic information about the involved extracellular, polyester degrading enzyme.

The main questions to be answered were:

What kind of enzyme is involved in the degradation of synthetic polyesters?

When is the enzyme produced by the microorganism?

What are the inducers of the enzyme activity?

What are the substrate specificities of the enzyme?

Based on the expected results for the enzyme of strain E11 it will be possible to make a comparison to other PCL or other synthetic polyester hydrolases and to set up of an adequate enzyme isolation and purification protocol.

3.9.1. Growth behaviour of strain E11 on GYM as complex medium and MSV supplemented with PCL (nanoparticles or films)

At the beginning the general growth behaviour for strain E11 which produces the extracellular enzyme is achieved. A series of experiments were carried out to study the growth behaviour for strain E11 in a mineral salts medium supplemented with PCL nanoparticles (0.1 % w/v) or films (one film /flask in 38 – 41 mg weight) as a sole carbon source and compare it with the growth on GYM as complex media. The results in Fig.3.16 show the growth was higher on GYM, and followed by the growth on PCL nanoparticles.

As expected, the growth was slowly on PCL films in comparison with the other experiments. The reduced growth is due to the smaller surface area of the films to be attacked by the microorganisms. In contrast, the organism grew much faster on nanoparticles than on the films due to their small size and larger surface area.

It was observed also from the results that the growth of strain E11 was similar on the all substrates A maximum of growth was obtained after 3 - 4 days. Therefore it can be expected that the growth on films increase when the same concentration from the carbon source was used. or also by carrying the experiments under the same conditions especially using the same volume from GYM medium in flask (250 ml) probably leads to increasing the growth on complex medium than both the other forms from PCL (see chapter 3. 9.3).

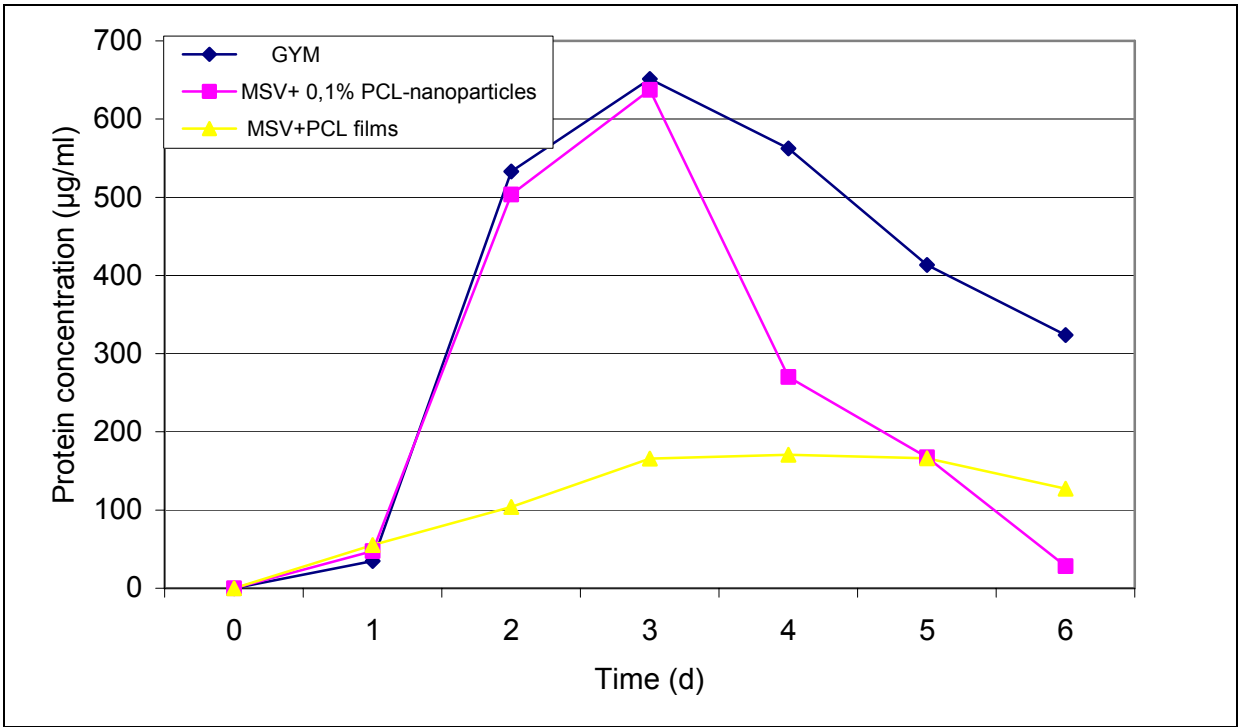


Fig. 3.16. Comparison between the growth behaviour for strain E11 on different media; GYM as complex medium and mineral salt media supplemented with PCL (nanoparticles or film)
Media: GYM complex medium (30 ml/flask (100 ml); Inoculum: 1 ml /flask (10^7 CFU/ml)
pH: 7
Mineral salt medium (MSV): 50 ml /flask (250ml); 3 ml/flask (10^7 CFU/ml)
Incubation at 30°C and 160 rpm,
Polymer: PCL nanoparticles (0.1 % w/v), Film: 25 mm in diameter, thick: 75µm, surface area: 9.82 cm²

3.9.2. PCL-hydrolase production from strain E11 in submerged culture with PCL (nanoparticles or films)

After having characterized the growth behaviour of the strain, the question was, at which stage of the culture the polyester degrading enzyme is produced? To answer this

question, E11 was grown on a mineral salts vitamin (MSV) medium supplemented with PCL (nanoparticles or films) as a sole carbon source and the activity of the produced enzyme was measured during the culture.

a- With PCL-nanoparticles

Fig. 3.17 illustrates that the extracellular PCL hydrolase formation started when the organism grew on PCL nanoparticles. The results demonstrated that, enzyme formation in submerged culture started after 2 days from the cultivation and after that the activity of the enzyme decreased at the end of the stationary phase. The enzyme activity disappeared, when all PCL was consumed by the organism. The PCL hydrolase activity was detected photometrically by decreasing in turbidity of PCL suspension ($OD650_{nm}$). To estimation the residual PCL in liquid medium, in parallel experiments the residual solids were centrifuged after the disappearance of enzyme activity , the pellets were washed with water 3 times, the pellets were dried at 30°C to weight constant, after that the pellets were analyzed by GPC. No PCL could be detected in the treatments with strain E11. On the other hand in the noninoculated control treatments showed 66% from PCL plus PCL particles were not unsoluable in the solvent probably this produced from aggregation PCL nanoparticles with salts in mineral salts medium.

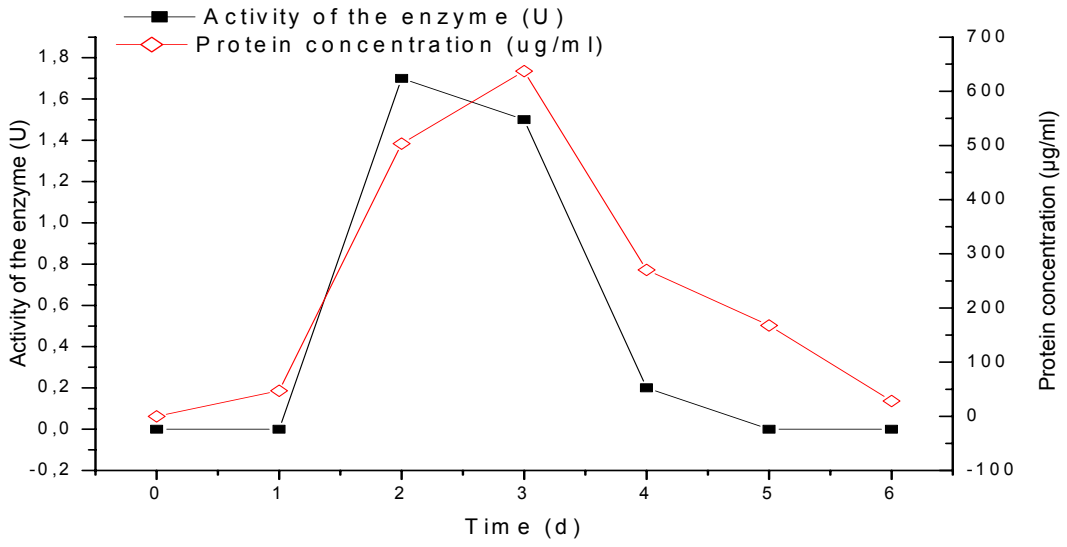


Fig. 3.17. PCL hydrolase production of E11 strain in submerged culture with PCL-nanoparticles.

Medium: MSV liquid medium, pH: 7, Temperature: 30°C, Shake at: 160 rpm, 50 ml/flask (250ml), Inoculum: 3 ml (10^7 CFU/ml). Measuring method: Decrease in turbidity of PCL suspension ($OD650_{nm}$), Incubation time for measuring the activity: 1h

Polymer: PCL- nanoparticles (0.1% w/v), n: 6 replicates

b- With PCL films

The results shown in Fig 3.18 demonstrated that the enzyme formation with the PCL-films started when the films decomposed into fragments after 3 days. The activity of the enzyme decreased then at the end of the stationary phase. The activity disappeared, when the PCL films were degraded completely. The activity was detected photometrically by decreasing in turbidity of PCL suspension ($OD650_{nm}$). PCL-films were degraded completely after 3-4 days in liquid mineral salts medium but the films were degraded about 90% at 30°C after 4 weeks with the same strain on solid agar medium. This difference in the degradation rate may be explained by the differences in cultivation conditions in both cases. In the liquid medium a good aeration can lead to an increase in biomass and the extracellular enzyme. Furthermore the enzyme can attack both surface of the polymer films.

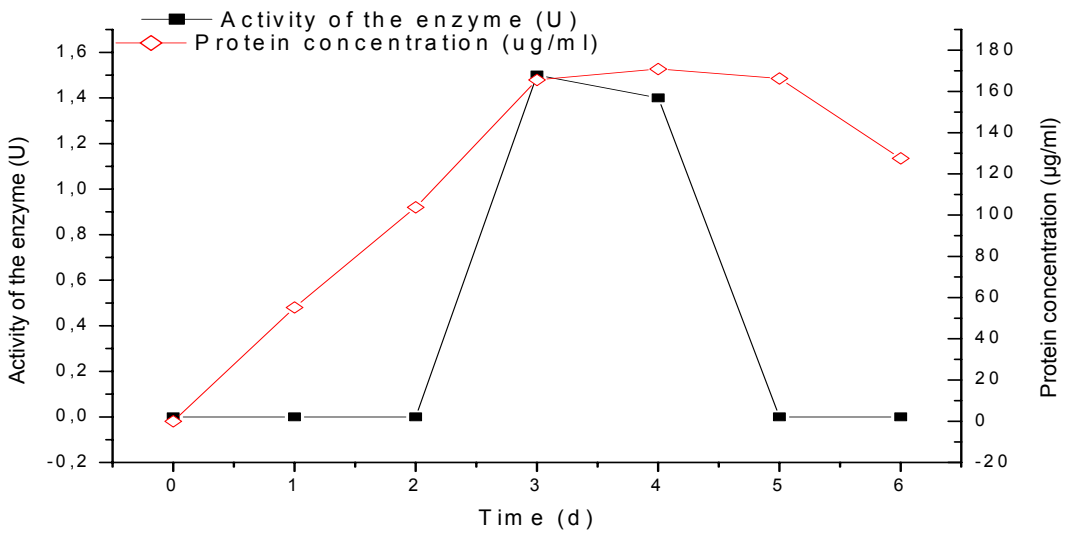


Fig. 3.18. PCL –hydrolase production of E11 strain in submerged culture with PCL-films.

Medium: MSV liquid medium, pH: 7, Incubation: at 30°Cand 160 rpm, 50 ml/flask, Inoculum: 3ml (10^7 CFU/ml), Measuring method: Decrease in turbidity of PCL suspension ($OD650_{nm}$), Incubation time for measuring the activity: 1h

Polymer: PCL film, diameter: 25 mm, thick: 75 µm, 1 film/flask, n: 10 replicates

3.9.3. Regulation of enzyme production (constitutive or inductive enzyme)

According to Schlegel (1992) most enzymes systems involved in substrate degradation are inductive enzymes. This means that the required enzymes are only produced and secreted in high amounts by the bacterial cell if the specific substrate is present in the direct surrounding. The question therefore arose, if the PCL degrading enzyme system is constitutively secreted or induced by the presence of PCL or other synthetic polyester? Furthermore it is of interest, if the enzyme is inducible, what are the substances inducing the enzyme activity?

A series of experiments were carried out to study the effect of media supplemented with different carbon sources on the growth of strain E11 and the hydrolase induction. GYM was used as complex media and it was supplemented with PCL (0.1% w/v as nanoparticles). A mineral salt vitamin (MSV) medium was supplemented with glucose (8 g/l^{-1}), PCL and BTA45:55 (Ecoflex) (0.1% w/v as nanoparticles) as carbon sources. All cultures were incubated at 30°C and 160 rpm for 6 days. Fig. 3.19A shows that the cell growth (determined as concentration of intracellular protein) was high on GYM, followed by GYM+PCL, MSV+ glucose, MSV+ glucose + PCL, MSV+PCL, MSV+BTA45:55 (Ecoflex), and latter on MSV without carbon source. The growth was slowly on BTA-copolyester in comparison with the other cultivations. When E11 was cultivated on glucose plus PCL in the same media, the cell growth was low in comparison to the culture in mineral salt medium supplemented only with glucose. This is probably due to a competitive inhibition for the two substrates.

Fig. 3.19b indicates that the extracellular hydrolase was produced only during growth of strain E11 with PCL and BTA 45:55 (Ecoflex) in mineral salt vitamin medium as carbon sources and may be these hydrolases are not similar. Enzyme formation started after 3 days from the cultivation and it decreased at the end of the stationary phase. After complete degradation of the PCL nanoparticles, the activity of enzymes disappeared. The activity was detected photometrically at OD_{650nm} by decreasing in turbidity of PCL suspension ($OD650_{nm}$).The results demonstrated that a maximum hydrolase activity was obtained when PCL was used as substrate. The low enzyme activities measured with BTA 45:55 (Ecoflex) as substrate, probably can be explained by enzyme adsorption on the hydrophobic surface of BTA 45:55 (Ecoflex) as still residual polymer is present in the medium Gouda et al. (2002). On the other hand in GYM as a complex media, in GYM+ PCL, MSV + Glucose, MSV+Glucose+PCL enzyme secretion is not induced despite the media generated a good cell growth.

The induction of hydrolytic enzymes by unsoluble polymers raises the question, how such a polymer passes through the outer cell membranes. Large structural materials, such macromolecules, cannot pass outer cell membranes. Therefore, the presence of polymers induces or enhances the microbial production of enzymes which excreted into the environment and are capable to cleave specific bounds in the polymer chain being available for the enzymatic system on the surface of the polymeric material. Thus, the solid polymer is destructed layer by layer and short chain and water soluble intermediates and monomers are generated, which can be assimilated into the cells. According to Lin and Kolattukudy (1978) microorganisms secret continuously low amounts of various extracellular hydrolyzing enzymes into their surrounding. The depolymerization products thereby produced are taken up into the cell, where they can induce the synthesis of appropriate amounts of the required or favorable hydrolyzing enzyme. The authors supported their hypothesis with investigations of the phytopathogenic fungi *Fusarium solani f. pisi*. The induction of cutinase by this fungal strain did not only take place in presence of cutin in the medium, but also the low levels of substrate hydrolysate which would consequently be generated then enter the cells and induce the synthesis of the enzyme. Cutinase activity is induced in the supernatant of *Fusarium solani f. pisi* and other fungal phytopathogenic fungi cultures in media containing cutin as a carbon source. The repression of the cutinase formation by glucose could be proven.

Murphey et al., (1996) found also that the presence of glucose in the PCL agar medium repressed degradation of PCL. Oda et al., (1995) showed also that the presence of either soluble starch, lactose or glucose repressed the synthesis of PHB and PCL depolymerase by *Paecilomyces lilacinus*. In the present work was found also that the PCL-hydrolase was induced in the culture supernatant with PCL or BTA 45:55 (Ecoflex) as substrates, but was not induced on glucose or GYM media.

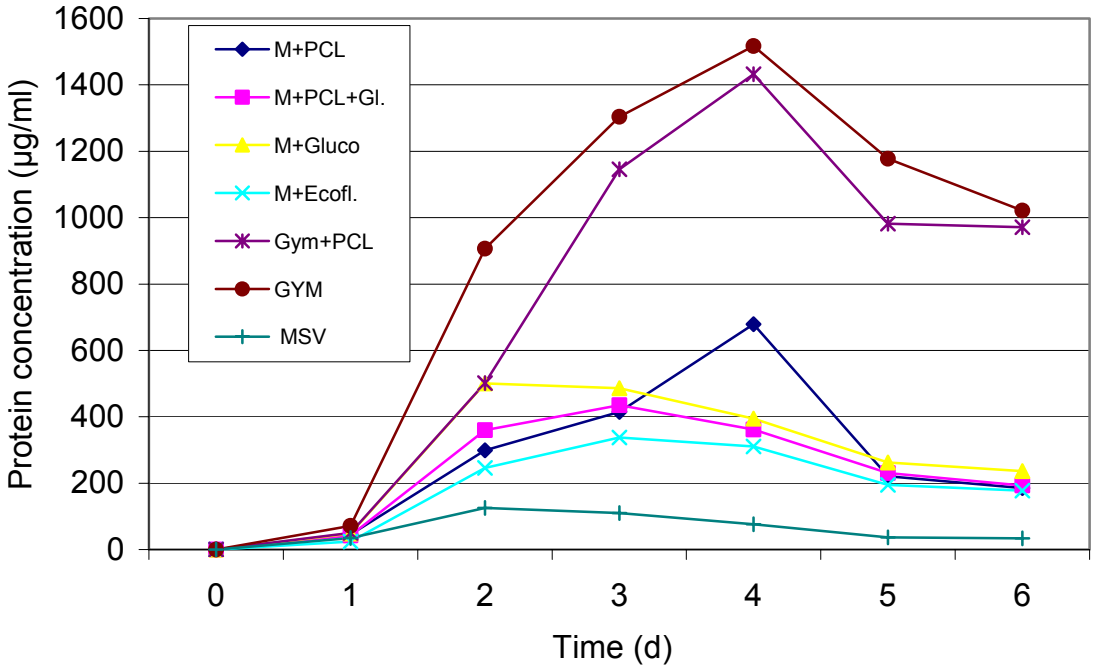


Fig. A.

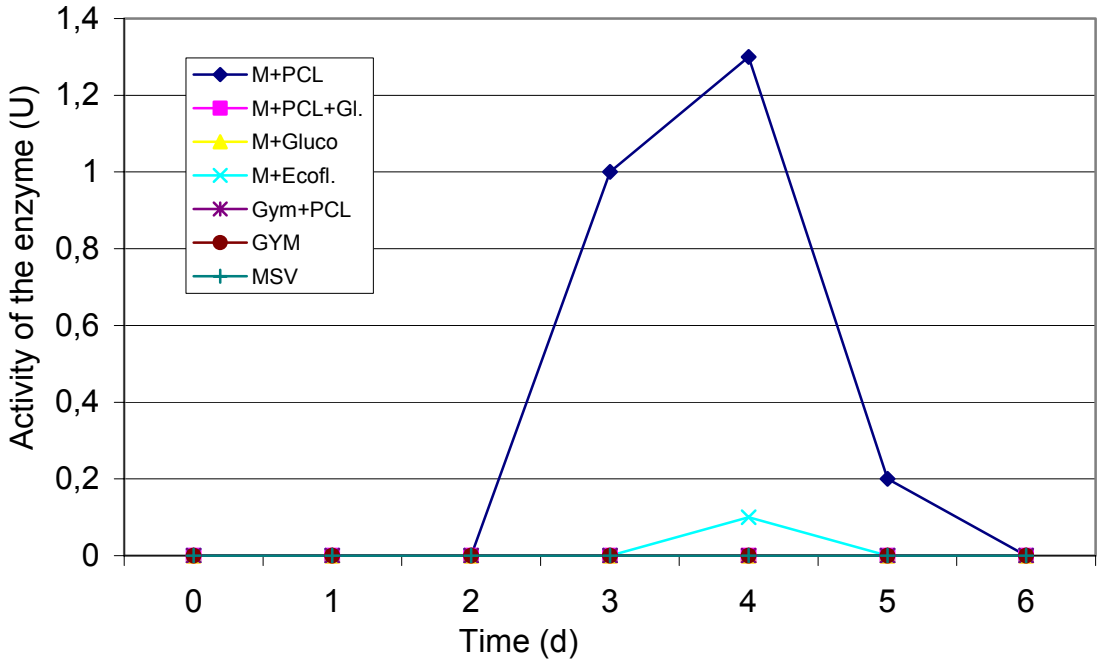


Fig. B

Fig 3.19. A. Growth curve of strain E11 on GYM and MSV supplemented with different substrates and B- PCL-hydrolase activity on the different substrates.

Medium: GYM and MSV liquid medium, pH: 7, Incubation at: 30°C and 160 rpm, medium volume: 50 ml/flask, Inoculum: 3ml (10^7 CFU/ml). Glucose: 8g/l, Measuring method: Decrease in turbidity of PCL suspension ($OD650_{nm}$), Incubation time for measuring the enzyme activity: 1h, Polymer: PCL nanoparticles (0.1% w/v), BTA45 :55 (Ecoflex)-nanoparticles (0.1% w/v), n: 3 replicates

3.9.4. Preliminary enzyme Purification studies

In the following, preliminary work to isolate and characterize the enzymatic system, responsible for the PCL depolymerization, is presented.

1. Ultrafiltration

Literature describes ultrafiltration as effective step for an initial purification since low molecular weight compounds can be separated depending on the cut off of the used membrane (Andersson, 1980). In addition ultrafiltration was successfully applied during purification of aerobic deplomyerases by Schirmer et al., (1993); Jendrossek et al., (1993B) and Müller and Jendrossek (1993). In this work the ultrafiltration process was carried with N_2 gas at 3 bar pressure in a cooling room at 4°C. Ultrafiltration process was carried out with a cut off of 10 KDa. After the ultrafiltration process protein bound on the membrane was found. The protein bound was washed with 5 ml from 20 mM phosphate buffer pH7. The activity was estimated photometrically by decreasing in turbidity of PCL suspension ($OD650_{nm}$). The mixture was incubated at 30°C for 30 minutes. The activity was determined as 1.5 U.

2. Enzyme purification and characterization by ion exchange chromatography

In order to isolate, purify and characterize a certain enzyme an appropriate enzyme activity detecting test must be available. The test should be highly selective towards the enzyme of interest, sensitive, fast and reproducible (Cooper, 1980). Several enzyme tests previously applied with PHB depolymerases (Jendrossek et al., 1993A; Müller and Jendrossek, 1993), a BTA hydrolase (Kleeberg et al 1999), and PCL hydrolases (Oda et al., 1997) are described in literature and some of these protocols proved to be suitable for the enzyme under investigations.

Purification of extracellular hydrolases from microorganisms have been extensively reported. Most of the purification procedures for enzymes were based on a combination of several non- specific techniques, such as ammonium sulphate precipitation, and ultrafilteration, gel filtration and ion-exchange chromatography. These methods were summarized in Table 3.12.

Table 3.12. In the literature methods for purification of enzymes

Enzyme	purification technique	Literature
Lipases	GF, GF	Taipa (1992) (review)
	ASP, GF, IEXC (K), IEXC (A)	
	UF, IEXC (A), HIC, IEXC (A)	
	ASP, IEXC (A), HIC	
	ASP, HIC, GF	
PHB-depolymerase	ASP, IEXC (A), HIC	Jendrossek et al. (1993)
	ASP, IEXC (A), IEXC (A), GF	Jendrosssek et al. (1995a)
	IEXC (A), HIC	Schirmer et al. (1995)
Cutinase	AP, IEXC (A), IEXC (A), HIC, GF	Sebastian and Kolatttukudy (1988)
Xylanasen	ASP, GF, IEXC (A)	Berens et al. (1996)
	IEXC (A), GF	Bachmann and McCarthy (1991)
Nylon hydrolase	IEXC (A)	Deguchi et al., 1998)
BTA hydrolase	A, HIC	Kleeberg et al., (1999)

A: anion exchange, AP: acetone precipitation, ASP: ammoniumsulfate precipitation, GF: gel filtration, HIC: hydrophbic interaction chromatography, IEXC: ion exchange chromatoraphy, K: cation exchange, UF: ultrafiltration.

The ion exchange chromatography was used in the present work because it was used by (Kleeberg et al., 1999) in the purification of BTA hydrolase which was produced by *Thermomonspora fusca* as actinomycetes.

The cultures were harvested (1800 ml) for the cultivation strain E11 with PCL, centrifuged at 11400 rpm for 20 min filtrated (RC 58 membrane filter, 0.2 µm, 47 mm Scleicher and Schüll, Dassel, Germany) and concentrated with a cutoff 10 kDa at 4°C to 30 ml with concentration of protein 1.42 mg/ml. After the ultrafiltration process the concentrated crude enzyme was filtrated (0.2 µm, Minisart, Sartorius, Göttingen, Germany). The Mono Q column was washed with 50 ml from 20 mM Tris/HCl-buffer pH9. 2 ml from the concentrated supernatant (protein concentration 1.42 mg/ml) was loaded onto a Mono Q column (9315048 Pharmacia) (Deguchi, et al., 1998), which was equilibrated with 13 ml from 20 mM Tris/HCl-buffer pH9. Afterwards the column was washed by with a linear gradient of 1mM NaCL in start buffer pH9 at flow rate of 0.3 ml/min (in total volume 40 ml). Many peaks were observed before and after the elution with a linear gradient of 1mM NaCL in start buffer pH9 (Fig 3.20). It was detected the activity in two states before and after the linear gradient with 1mM NaCL in start buffer pH9 of the column. The PCL hydrolase activity was detected photometrically at OD_{650nm} during the chromatographic run by decreasing in turbidity of PCL suspension ($OD650_{nm}$).

All fractions with positive activity were analyzed with SDS-PAGE. In most cases before and after by applying a 1 M NaCl in start buffer gradient was observed several protein bands in gel per fraction. On the other hand two fraction after applying a 1 M NaCl in start buffer as gradient appeared one protein band in gel. Fig. 3.21 shows one protein band for the purified protein SDS-PAGE.

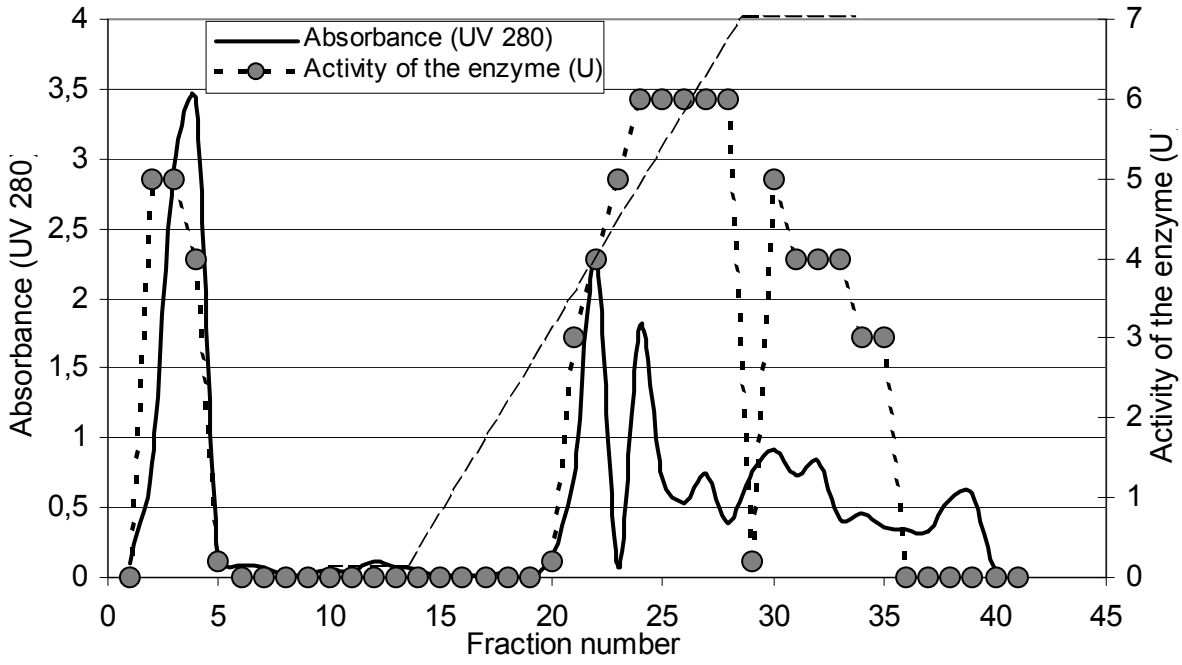


Fig. 3.20. Anion exchange chromatography with Mono Q column

Start buffer: 20mM Tris/HCl pH 9.0 Elution: 1 M NaCl in start buffer

Sample: 2 ml culture supernatent after ultrafiltration (1.42 mg/ml)

Flow rate : 0.3 ml/min,

Measuring method: Decrease in turbidity of PCL suspension ($OD650_{nm}$) Time: 5 min.

Fraction volume: 0.9 ml

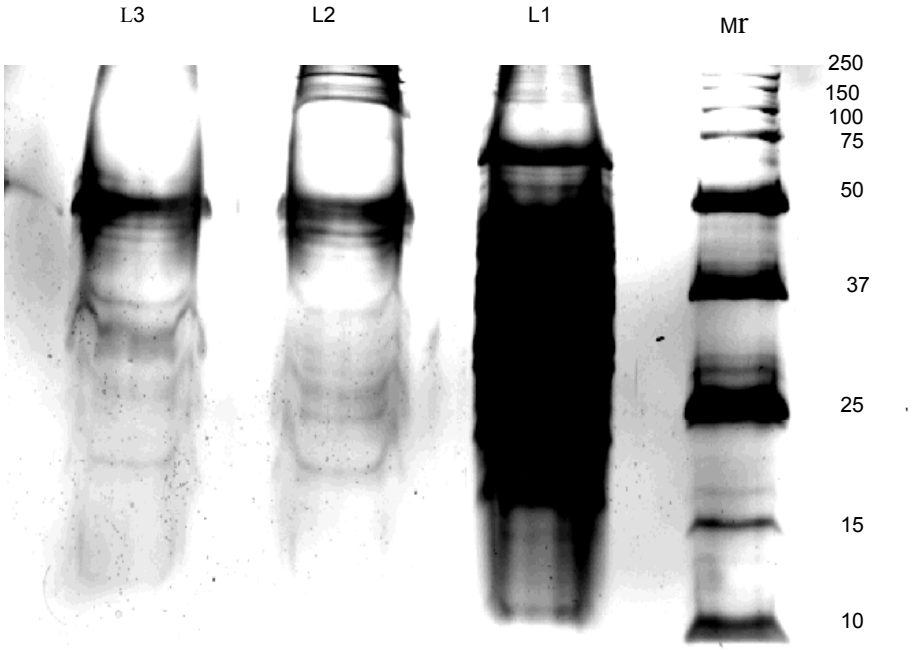


Fig. 3.21. SDS-PAGE with PCL-hydrolase after elution with Mono Q column with the active fraction.

Mr molecular weight standard (kDa), L1 concentrate sample after ultrafiltration process, L2 and L3 two fraction after elution both contained one band.

400 µl of the fractions containing one band (from L3 in Fig 3.27) were loaded on a Q column as ion exchange (according to the protocol of the Vivapure company; see Materials & Methods).

The Q column was equilibrated with 400 µl from 20 mM Tris/HCl pH9 as buffer A. 400 µl from the sample which was contained one protein band (L3 in Fig 3.27) were loaded on the column. The column was washed with 400 µl from 20 mM Tris/HCl and this step was repeated for another time. After that the column was eluted with 400 µl from 1 M NaCl in Tris/HCl pH9. The activity was determined photometrically by decrease in turbidity of PCL suspension ($OD650_{nm}$) in all fraction. The activity was detected before and after the elution of Q column. The factions with positive activity was analyzed with SDS-PAGE in Fig 3.22.

The results shown in Table 3.13 summarizes the purification steps with both column (Mono Q and Q) for PCL hydrolase.

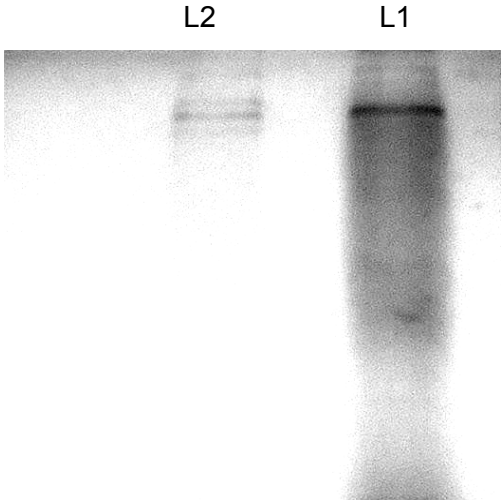


Fig. 3.22. SDS-PAGE with PCL -hydrolase after elution from Q column
L1 active fraction before elution, L2 active fraction after elution

Table 3.13. Partial purification of PCL- hydrolase from culture fluid of E11 strain

Sample	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity U/mg	Protein recovery %
Culture fluid	1800	196.56	2340	11.9	100
Concentrate	30	42.6	720	16.9	30.8
Mono Q	0.9	0.182	5.4	29.7	0.231
Q_1	0.4	0.0522	2.4	46	0.102
Q_4	0.4	0.0246	1.6	65.04	0.1

Q_1 before elution for Q column

Q_4 after elution for on Q column

3. Activity detection of the purified enzyme with native gel electrophoresis

The activity of the isolated protein band was detected by clear zone formation on native polyacrylamide gel (5%) containing 0.1% PCL as substrate for enzyme activity testing. The purified sample (Q4) (concentration of protein 0.0246 mg/ml) was applied on native polyacrylamide gel. After electrophoretic treatment, one gel was separated and was stained with commasie blue to determined the position of protein band and clear zone formation on gel. Fig 3.23 show the appearance of a single zone of clearing on the native polyacrylamide gel which incorporated with PCL suspension (0.1%). This technique was

used by many authors (Magnuson and Crawford 1997, Ruiz et al., 1999, Abou-Zeid et al., 2001).

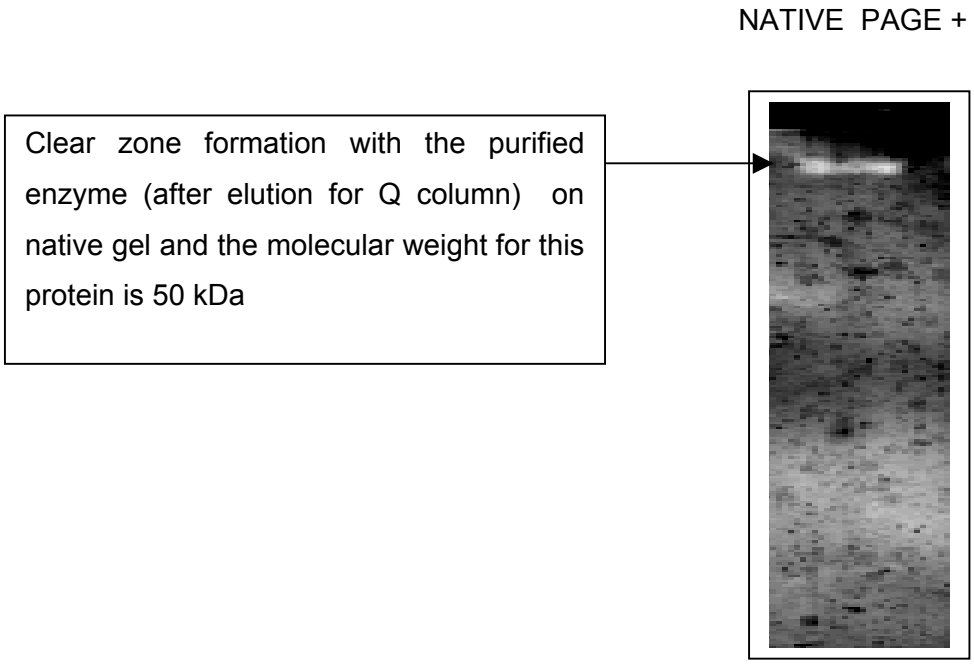


Fig.3.23. Native polyacrylamide gel electrophoresis (5%) with 0.1 % PCL-suspension directly incorporated into the gel matrix. Clear zone formation with the purified enzyme (after elution with Q column) on native gel after incubation 15 h at room temperature. The gel was stained with commasie blue.

Further attempts to purify PCL hydrolase with other methods were not performed due to the time limitations.

3.9.5. Determination of substrate specificities of the crude PCL- hydrolase

1. Via estimation of diameter Clear zone on MSV-agar containing the polyesters (0.1% w/v) at 30°C

Degradation of different synthetic and natural polyesters was investigated with *Microbispora rosea subsp. rosea* (strain E11) on agar plates and with weight loss determination of polymer films on agar plates (chapter 3.2.2.2 and 3.3). The question remained open whether the organism produced different hydrolytic enzymes for individual polyester structures or whether one substrate specificity has the PCL hydrolysing enzyme sufficiently broadly to degrade all structures. The PCL hydrolase as concentrated crude enzyme after ultrafiltration was tested with emulsified BTA 45:55 (Ecoflex), BTA 40:60, PCL, SP46 as well as PHBV in mineral salt vitamin agar medium. 100 µl from the concentrated crude enzyme (protein concentration 0.94 mg/ml) was put in a well on

emulsified mineral - salt -vitamin agar with the polyesters (0.1% w/v). and the plates were incubated at 30°C for 15 h. The results in Table 3.14 show that the PCL-hydrolase was able to form clear zones with synthetic polyesters including BTA-copolyester (BTA 45:55, BTA 40:60), SP 4/6 and PCL. The general trend in diameter of clear zones for the synthetic polyesters was SP 4/6>PCL>BTA 40:60>BTA 45:55 (aliphatic homopolyester > BTA–copolyester). The PCL-hydrolase has therefore a broad substrate specificity for synthetic polyesters. No hydrolytic activity was observed for PHBV as natural copolyester by the PCL-hydrolase. However, PHBV films were degraded by strain E11 on agar plates. This proves the ability of strain E11 to produce at least two different hydrolases, a PHB-depolymerase and a PCL-hydrolase. PCL is degraded by lipases and esterase's (Tokiwa and Suzuki 1977) and a PHB depolymerase did not hydrolyze PCL (Jaeger et al., 1995). Therefore, probably the PCL-hydrolase is a cutinase or lipase but no PHB depolymerase.

Table 3.14. Substrate specificities of PCL-hydrolase on MSV-agar plates supplemented with synthetic or natural polymers (0.1%) after 15 h at 30°C.

Polymers	Clear zone (diameter mm)
1- Synthetic polymers	
BTA 45:55 (Ecoflex)	(7mm)
BTA 40:60	(10 mm)
PCL	(15 mm)
SP 4/6	(20 mm)
2- Natural polymers	
PHBV	0

2. Substrate spectrum of the crude PCL-hydrolase concerning fatty acids (triglyceride)

The natural substrates of lipase are triglyceride of long-chain fatty acids. These triglyceride are insoluble in water and lipases are characterized by the ability to rapidly catalyse the hydrolysis of ester bonds at the interface between the insoluble substrate phase and the aqueous phase in which the enzyme is soluble. A number of different substrates have been used for the assay of lipases, however the preferred methods use emulsions of insoluble triglyceride. Stable emulsions of triglyceride were prepared (see

Materials & Methods) and was used to test the activity for the enzyme under investigation. The progress of the PCL-hydrolysis is normally followed by measurements of the acids released fatty acids are titrate with NaOH by using a pH-stat autotitrator. The rate of ester cleavage was calculated by linear regression from the slope of the plot of consumed NaOH versus time. The activity of PCL-hydrolase towards the mixture from fatty acids was 0.52 U/mg^{-1}. It was observed that the activity for crude PCL hydrolase was about 9.6 and 40 fold smaller than hydrolase from *Thermomonspora fusca* and lipase from *Candida cylinderacea*, respectively Welzel et al., (2002) probably due to increasing the purified protein content in both enzymes (hydrolase from *T. fusca* and lipase from *Candida cylinderacea*). The activity was calculated from the slop of titration curve in Fig. 3.24. All lipases and cutinase hydrolysed triglyceride (Jaeger et al., 1995). Therefore, probably the PCL-hydrolase is cutinase or a lipase but not a PHB –depolymerase.

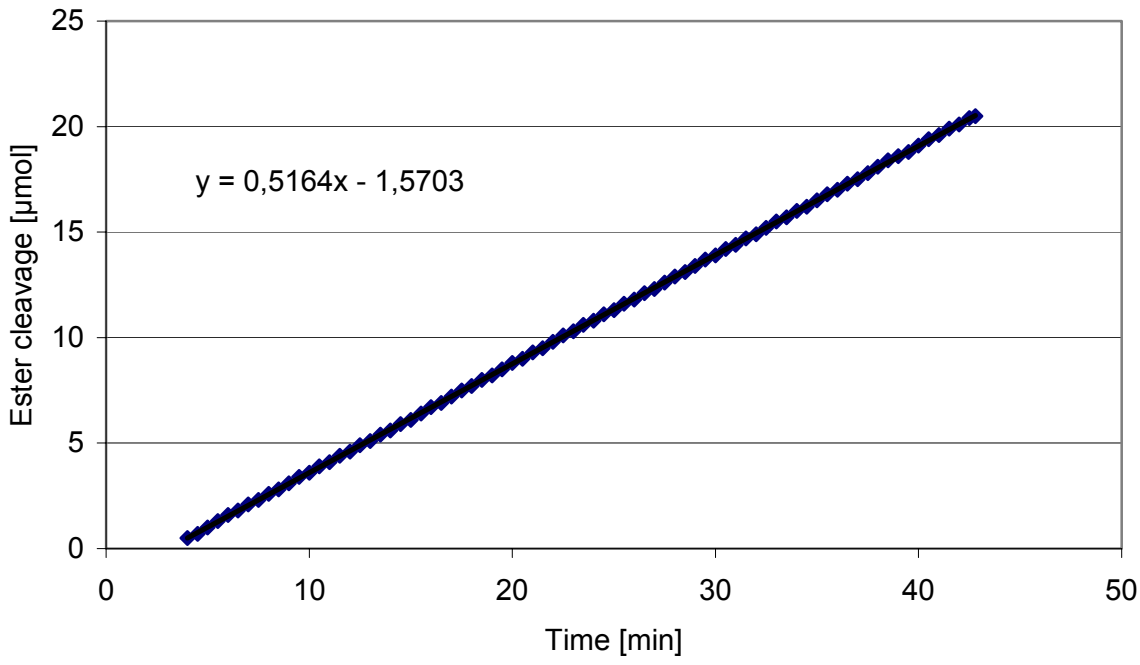


Fig.3.24. PCL -hydrolase activity for fatty acids by titration methods

Crude PCL-hydrolase volume: 500 µl, concentration of protein: 1.96 mg/ml, Temperature: 30°C, Fatty acids: mixture from Triolein and Triacetin, measuring method: titration method

3.9.6. Effect of pH on the crude PCL-hydrolase activity (optimum pH)

This buffer system was used earlier by (Kleeberg et al 1999) to study the effect of pH on the activity for hydrolase from *Thermomospora fusca*. For the determination of the optimum pH of the PCL-hydrolase activity different buffer systems were used. For a pH range of 4 to 7 a 150 mM citrate buffer was used. The pH values of 5.5 to 8 were prepared with 20 mM phosphate buffer and the remaining pH value from 5.5 to 8 were obtained using 100 mM phosphate buffer, PCL hydrolase was used at a protein concentration 0.92 mg/ml, and the measuring time was 5 min. The activity for PCL hydrolase at different pH was measured by using OD_{650nm} technique. The optimum pH for PCL hydrolase was at pH =7 with both two phosphate buffer (20 and100 mM) in Fig. 3.25. Yet, a second optimum pH appeared at a pH 6 with citrate buffer (150 mM). It is known also from (chapter 3.4.2.1) that the optimum pH for growth the strain E11 on mineral salt medium with glucose was pH7. This buffer system was used earlier by (Kleeberg et al 1999) to study the effect of pH on the activity for hydrolase from *Thermomospora fusca*. The author found that pH optimum for hydrolase from T. fusca was 6.5 with both phosphate buffer (20 and 100 mM) and the second optimum pH was at 6 with citrate buffer (150 mM).

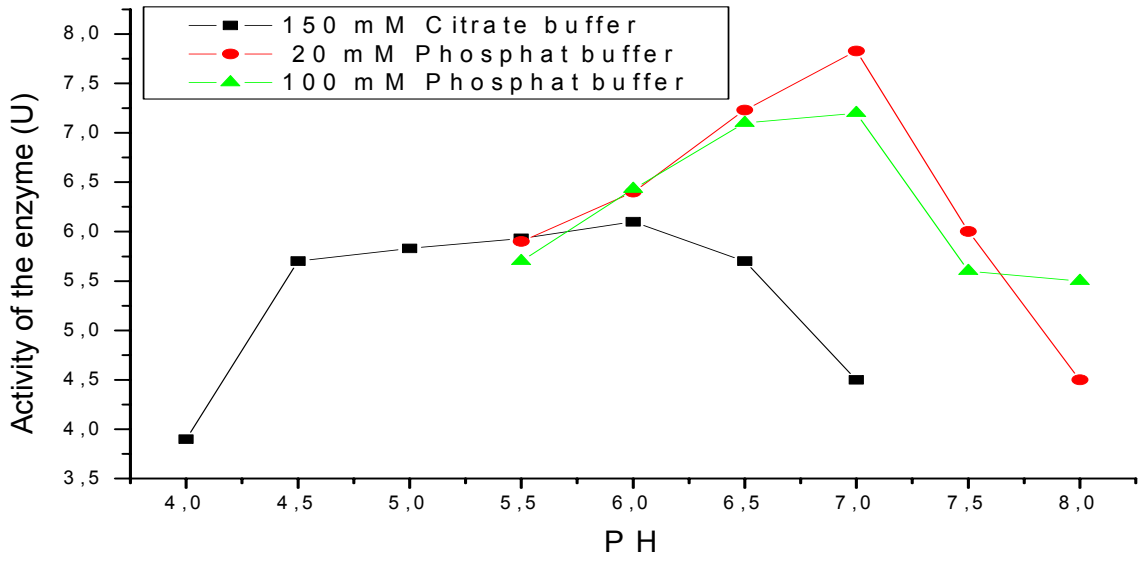


Fig. 3.25. Effect of different pH on activity of PCL- hydrolase

Protein concentration of crude enzyme: 0.92 mg/ml, Temperature: 30°C, incubation time: 5 min. Measuring method: Decrease in turbidity of PCL suspension ($OD650_{nm}$)

3.9.7. Storage of the enzyme

Normally, concentrated enzyme solutions can be stored at -20°C. However, crude enzyme extracts as those used during the following part of the work normally require storage temperatures between –20°C and –70°C (Cooper, 1980). The crude PCL hydrolase was stored from 72 h till 3 months at –20°C ; thawing of the crude enzyme and was used for further investigation. The stability of the enzyme under different preservation times is characterized in the following. A solution taken from the medium (1400 ml) and concentrated by ultrafiltration to 20 ml (protein content 0.92 mg/ml). The activity was estimated photometrically by decrease in turbidity of PCL suspension at OD_{650nm}. The activity was before the storage 20.3 U. After the storage at –20°C for 72 h and 3 months. The results in Fig 3.26 show that the activity was decreased 34.5 and 65.5% after 72 h and 3 months respectively. By comparison with other enzymes such as hydrolase from *T. fusca* it was found 99.8 and 99.3% of the initial activity for hydrolase after storage at –20°C for 70 and 240 days, respectively (Gouda et al., 2002).

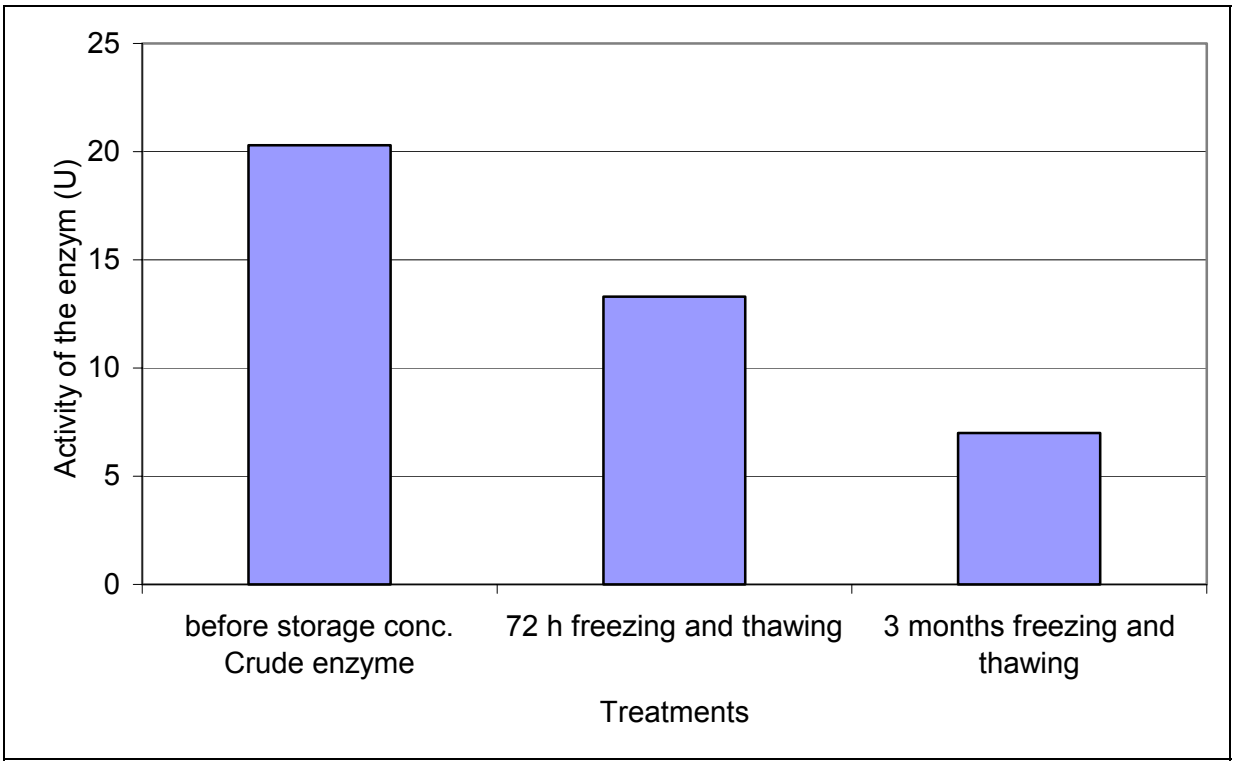


Fig 3.26. Effect of time storage on the activity of crude PCL hydrolase
Measuring method: Decrease in turbidity of PCL suspension ($OD650_{nm}$),
Protein concentration: 0.92 mg/ml
Temperature: 30°C, incubation time: 5 min.

3.9.8. Stability at different temperature

For determination of the thermal stability, the concentrated crude PCL hydrolase (with concentration of protein 0.92 mg/ml) was incubated at different temperatures from 15 to 80°C for 10 min. PCL hydrolase activity was then measured at 30°C for 5 min by Decrease in turbidity of PCL suspension ($OD650_{nm}$). The results shown in Fig. 3.27 clearly indicate a general sensitivity of the enzyme towards high temperatures. The enzyme activity was stable from 30°C till 60°C as a result of exposure to high temperature, where the enzyme was not stable at 70 and 80°C. It is known from (chapter 3) the strain E11 can grow at range from 25 to 40°C and the optimum temperature was 30°C.

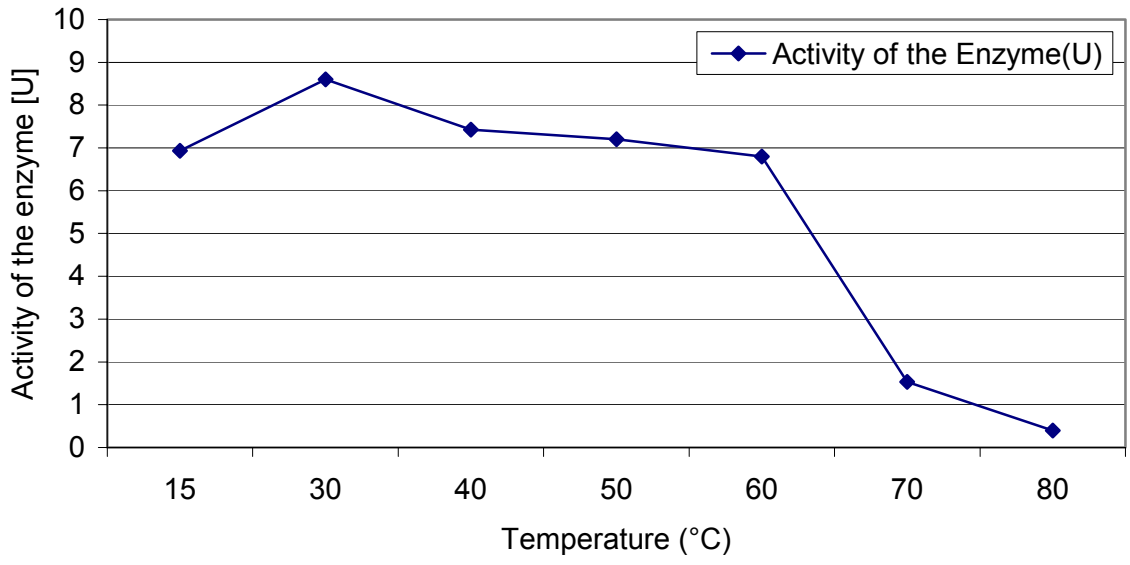


Fig. 3.27. Stability of PCL- hydrolase at different temperatures

Protein concentration in the crude enzyme: 0.92 mg/ml, Incubation at different temperature: 10 min, measuring time: 5 min at 30°C. Measuring method: Decrease in turbidity of PCL suspension ($OD650_{nm}$),

4. Conclusive Discussion

4.1. Isolated organisms

Studies on the biodegradation of synthetic and natural plastics were performed by isolation of different microorganisms under mesophilic conditions. A total of 47 different aerobic degrading strains which have the ability to degrade the synthetic and natural polyesters were isolated from different habitats (mature compost 6 months old and enrichment garden soil cultures with PCL or BTA 45:55 (Ecoflex)). These isolates are embraced fungi, actinomycetes and bacteria. Five polyester degrading fungal strains were identified according to colony and morphological characteristics as *Arthrobotrys amerospora* (EB1), *Acremonium strictum* (EB14), *Fusarium solani* (EB10), *Aspergillus fumigatus* (EB19), and *Cladosporium herbarum* (EB2T). Three actinomycetes strains were identified according to the morphological, physiological, chemotaxonomical (fatty acids clusters) as well as molecular genetic (16 rDNA), two of those strains belong to *Microbispora rosea subsp. rosea* and one as *Streptomyces thermocarboxydus.* Many synthetic or natural polyesters degraded by fungal, actinomycetes and bacterial isolates were isolated by many authors under different conditions (Bendict et al., 1983, Mergaert et al., 1994, Foster et al., 1995, Jaeger et al., 1995, Oda et al.,1995, Schirmer et al., 1995, Kawai 1996, Mergaert and Swings 1996, Roßhaupten and Molitoris1996, Murphy et al., 1996, Oda et al., 1997, Kleeberg et al., 1998, Nishida et al., 1998, Gödde, et al., 1999, Nishide et al., 1999, Scherer et al., 1999, Ruiz et al., 1999, Kim et al., 2000, Uchida et al., 2000, Orhan and Büyükgünger 2000; Abou Zeid et al., 2001; Rowe and Howard 2002).

In the present study the PCL as aliphatic homopolyester was degraded faster than aliphatic/aromatic BTA- copolyester by most isolates. This behaviour was expected from data found in the literature. Tokiwa and Suzuki (1981) reported that the melting point is an important factor in the biodegradability of the polyester where the biodegradability decreased with increasing the melting point. Marten (2000) postulated for instance that the reduced flexibility of the copolyester chains, which correlates with the relative high melting points of the copolyesters, hinders the polymer chains to adjust into the active site of the hydrolyzing enzymes.

On the other hand BTA copolyester was degraded faster than the aliphatic polyester PCL by fungal strain EB1. This strain was identified as *Arthrobotrys amerospora.* It can be anticipated, that this organisms and its extracellular enzymes prefer hydrophobic surfaces as offered by the BTA-copolyesters. Kleeberg et al., (1998) found previously that BTA 40:60

was depolymerized much faster than aliphatic polyesters such as Bionolle, Bayer Tir 1874 and SP313 under thermophilic conditions with two strains from *Thermomonspora fusca*.

4.2. Classification of the polyester degrading isolates depending on their substrate (polyester) specificities

The polyester degrading strains isolated throughout in this work were divided into two separate groups depending on their substrate (polyester) specificities.

4.2.1. Strains degrading only synthetic polyesters

These strains seems to be specialized only on degradation the synthetic polyesters (BTA-copolyester (BTA 45:55 (Ecoflex) and BTA 40:60, PCL, SP 4/6) and none of them could attack the natural copolyester such as PHBV. These isolates were identified as *Arthrobotrys amerospora* (EB1), *Acremonium strictum* (EB14), *Cladosporium herbarum* (EB2T) and *Gliocladium roseum* (G1) as fungal strains.

4.2.2. Strains degrading natural and synthetic polyesters

This group shows a wider substrate spectrum since they exhibited depolymerization activity towards the synthetic and natural polyesters under investigation. They include fungi and actinomycetes. The fungal isolates were identified as *Fusarium solani* (EB10) and *Aspergillus fumigatus* (EB19) as well as one strain from *Gliocladium roseum* (G2). The actinomycetes isolates are *Microbispora rosea subsp. rosea* (E11 and Act3 strains) and one is as *Streptomyces thermocarboxydus.*

Obviously, at least three different enzyme systems are discussed to be involved in the aerobic degradation of the different polyesters where these different types of enzymes are PHB depolymerases, lipases and cutinase. Non of the PHB depolymerases shows significant lipase activity or hydrolyze synthetic polyesters (Jaeger et al., 1995). However, many lipases depolymerize synthetic polyesters such as PCL, but cannot depolymerize PHB (Pranumuda et al., 1995). Cutinases on the other hand, are serine hydrolases for primary alcohol esters (Kazlauskas, 1994; Svendsen 1994, Murphy et al., 1996) which hydrolyze cutin specifically, and PCL probably due to structural similarities between their depolymerization products.

Therefore, the organisms which are specialized only on degradation the synthetic polyesters due to these organisms secrete lipase or cutinase enzymes. On the other hand the organisms which depolymerize synthetic and natural polyesters as well, probably secrete at least two different enzymes, which are depolymerase and either lipase or cutinase.

4.3. Degradation of BTA 45:55 (Ecoflex) and PCL under natural conditions

The fungal strains play an important role in the degradation of the two polyesters (BTA 45:55 (Ecoflex) and PCL) under natural conditions (low temperatures). The degradation potential by the fungal strains differed by difference of the strains, the incubation temperature and the type of the polyesters.

Two strains which were identified as *Arthrobotrys amerospora* (EB1), and *Gliocladium roseum* (G1) can degrade BTA 45:55 (Ecoflex) films completely at 25°C. These isolates prevail over the other isolates with regard to their degradation rate. At the same temperature the degradation potential with *Arthrobotrys amerospora* (EB1) against PCL is lower than the other isolates and in the same time lower than BTA 45:55 (Ecoflex) because PCL was degraded slowly than BTA 45:55 (Ecoflex) at 25°C by this strain.

The strains *Arthrobotrys amerospora* (EB1) and *Cladosporium herbarum* (EB2T) degraded BTA 45:55 (Ecoflex) faster than the other isolates at 15°C. In contrast the isolates degraded PCL slower than the other isolates.

Three strains which have identified as *Fusarium solani* (EB10), *Acremonium strictum* (EB14) and *Cladosporium herbarum* (EB2T) play an outstanding role in the biodegradation for BTA 45:55 (Ecoflex) and PCL films at 4°C and these strains degrade the two polyester films more than the other strains at 4°C. It was used the phytopathogenic fungi by many authors in degradation of the synthetic polymers such as polycaprolactone (PCL), where the isolates secrete cutinase that degrades cutin (the structural polymer of the plant cuticle) (Oda et al., 1995, Murphy et al., 1996, Nishida et al., 1999).

It is known the phytopathogenic microorganisms secrete cutinase which degrade cutin specifically under different environmental conditions (Purdy and Kollattukudy 1973, 1975, Ettinger et al., 1978, Lin and Kolattukuddy 1980, Köller and Parker 1989 Fett et al., 1992 and 1994, Fan and Köller 1998, Cunha et al., 2003).

Not all fungal isolates can degrade the synthetic polyester under low temperatures despite their growth under the same conditions. The strain EB19 can grow at low temperatures on mineral salt vitamin medium with glucose but can not degrade the synthetic polyester under the same conditions and this is probably due to fact that the strain does not secrete the hydrolytic enzyme at the low temperature. In contrast, the other isolates grew at low temperature and simultaneously can degrade the synthetic polyesters.

Obviously the degradation rate depends on the fungal strain, type of the polyesters and also the incubation temperature probably due to these strains produce different hydrolytic enzymes at the different temperatures.

Degradation of BTA 40:60 a copolyester was investigated earlier under thermophilic conditions by (Kleeberg et al.,1998). By comparison between the degradation rate under thermophilic conditions with *T. fusca* and mesophilic conditions in the present work, the

degradation rate under thermophilic conditions was about 17.5 fold more than under mesophilic conditions.

The depolymerization of PCL was investigated previously under anaerobic conditions by Abou-Zeid et al., (2001). The authors showed that PCL films were depolymerized by strain *Clostridium acetobutylicum* at 37°C. By comparison between the degradation rate for PCL films by *Clostridium acetobutylicum* and the degradation rate with *Fusarium solani* and *Acremonium strictum* at range from temperature 4-30°C in the present work, the degradation rate was with *Fusarium solani* and *Acremonium strictum* at range from temperature 4-30°C about 10-73 fold higher than under anaerobic conditions.

4.4. Improved test system for BTA 45:55 (Ecoflex) degradation with *Arthrobotrys amerospora* (EB1)

Using BTA 45:55 (Ecoflex) nanoparticles and powder in degradation tests, *Arthrobotrys amerospora* (EB1) was successfully used in improved and significantly accelerated degradation tests. Unexpected BTA 45:55 (Ecoflex) powders were degraded rapidly than nanoparticles and these results was contradictive with previous details probably due to aggregation of the nanoparticles after the cultivation and they became large particle sizes and small surface area; difference of the incubation temperature and the type of the inoculm where powder experiments were incubated at 30°C and inoculated with spore suspension, on the other hand nanoparticles experiments were incubated at room temperature and inoculated with discs from complex medium (potato dextrose agar) bearing the fungal mycelium and may be existence these discs in the cultivation medium suppressed the enzyme production.

4.5. PCL degrading, aerobic enzyme system from strain E11

Experimental results indicate that the initial PCL breakdown is catalyzed by an extracellular PCL hydrolase which is induced after growth *Microbispora rosea subsp. rosea* strain E11 on PCL. As mentioned before, the PCL hydrolase synthesis was repressed during growth of strain E11 on glucose or GYM as complex medium in the presence PCL.

It remains questionable how an insoluble polymer such as PCL which cannot enter the cell, is able to induce PCL depolymerizing enzyme activity. After Lin and Kolattukudy (1978) microorganisms secret continuously low amounts of various extracellular hydrolyzing enzymes into their surrounding. The depolymerization products thereby produced are taken up into the cell, where they can induce the synthesis of appropriate amounts of the required or favorable hydrolyzing enzyme.

Similarly, literature on aerobic PCL-degradation gives evidence about the involvement of enzyme in PCL depolymerization (Oda et al., 1995, Murphy et al., 1996). They showed that PCL depolymerase was induced in the presence of PCL in the surrounding milieu and was repressed in the presence of either glucose, soluble starch, or lactose. Also Murphy et al., (1996) presented genetic, regulatory and enzymatic evidence for the involvement of cutinase in PCL degradation. In addition, they showed that PCL trimers are structurally similar to cutin monomers which are inducers of cutin activity.

The isolated PCL-hydrolase was preliminary characterized. The purified enzyme sample resulted in one molecular weight protein band 50 KDa. The optimum pH for crude PCL hydrolase was 7 with two phosphate buffer (20 and 100 mM). A second optimum appeared pH 6 with 150 mM citrate buffer. After incubation at 10 min PCL-hydrolase was stable up to about 60°C. PCL-hydrolase was active towards mixture from triglyceride (triolein and triacetin).
The concentrated crude PCL hydrolase exhibited broad substrate specificity towards the different synthetic polyesters via clear zone formation. While it can degrade the BTA-copolyester (BTA 45:55 (Ecoflex) and BTA 40:60), PCL and SP 4/6. It did not degrade PHBV. In literature, PCL is degraded by lipases and esterases (Tokiwa and Suzuki 1977). PHB depolymerase did not hydrolyze PCL (Jaeger et al., 1995). The substrate specificity for PCL hydrolase was studied also towards mixture from triglycerides by titration of free acids with NaOH which produce from triglyceride cleavage by pH-stat titration. All lipases and the esterase hydrolyzed triglyceride (Jaeger et al., 1995). Therefore, probably PCL-hydrolase is esterase, cutinase or lipase but no depolymerase.

The questions are still open, the purification for PCL hydrolase is not clear as well as isolation and purification of the degrading enzymes from fungal strains at different temperatures and comparison between these hydrolytic enzymes by their molecular weight, degradation activities, substrate specificities as well as analysis of the amino acid sequence.

5. Summary

The biodegradation of synthetic polyesters such as poly(tetramethylene adipate – co – tetramethylene terephthalate) (BTA copolyesters), poly(ε-caprolactone) (PCL) and polyteramethyleneadipate (SP 4/6) as well as the natural copolyester poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) was investigated under mesophilic conditions. By using a method based on clear zone formation on agar plates, a total of 47 polyester degrading strains (15 fungi, 24 actinomycetes, 8 general bacteria) could be isolated from mature compost and gardening soil using PCL and BTA 45:55 (Ecoflex) as substrate. Eight of the most efficient polyester degrading strains were selected and identified for further studies. Defined and accelerated degradation tests were established with these strains. The production of an inducible extracellular PCL hydrolase by strain E11 in liquid mineral salt medium was proofed and the hydrolase was characterized with regard to their basic properties.

The isolated polyesters degrading strains were classified according to their specificity for their degradation substrate into two groups. The first group are strains specialized to depolymerize only the synthetic polyesters (BTA-copolyesters, PCL and SP 4/6) but none of these isolates degraded the natural copolyester PHBV. From this group, selected fungal strains were identified as *Arthrobotrys amerospora* (EB1)*, Acremonium strictum* (EB14)*, Cladosporium herbarum* (EB2T) and *Gliocladium roseum* (G1).

The second group comprises strains which exhibited a wider substrate spectrum as they are able to degrade synthetic as well as natural polyesters. These strains include fungi and actinomycetes. Selected fungal strains were identified as *Fusarium solani* (EB10), *Aspergillus fumigatus* (EB19) and *Gliocladium roseum* (G2). A selection of actinomycetes strains were identified as *Microbispora rosea subsp. rosea* (isolates E11 and Act3) and *Streptomyces thermocarboxydus*.

PCL films were degraded faster than BTA 45:55 (Ecoflex) by most isolates as it was expected from results reported in the literature. However, the strain *Arthrobotrys amerospora* (EB1) degraded the aromatic-aliphatic copolyester BTA 45:55 (Ecoflex) faster than PCL (at 25°C), indicating, that this microorganism probably has an enzyme system specialized on the specific structure of these copolyesters.

With 7 fungal strains, comparative degradation tests with BTA 45:55 (Ecoflex) and PCL films were performed on agar plates at different temperatures (30°C, 25°C,15°C and 4°C). A different behavior concerning the temperature dependence was observed for these strains. While most of the isolates exhibited the most rapid degradation at higher temperatures, three fungal strains showed also significant degradation potential for the polymer films at 4°C.

These strains were identified as *Fusarium solani* (EB10), *Cladosporium herbarum* (EB2T) and *Acremonium strictum* (EB14).
The fungal strain *Arthrobotrys amerospora* (EB1) was used to perform degradation experiments with BTA 45:55 (Ecoflex) in a liquid mineral medium. Powder and nano-particles of the copolyester could be degraded up to 80% with this mesophilic strain within 12 weeks.

Extracellular PCL depolymerizing enzymatic activity in the supernatant of a culture from the strain *Microbispora rosea subsp. rosea* could be identified.
It was shown that the extracellular hydrolyase activity was induced by the presence of PCL and BTA-copolyester, while the presence of additional carbon sources such as glucose or a complex medium suppressed enzyme formation. The concentrated crude PCL-hydrolase was active towards triglycerides (triacetin and triolein) and a wide range of synthetic polyesters (BTA copolyester, PCL and SP 4/6). In contrast the enzyme was not capable to depolymerize the natural copolyester PHBV, although the organism itself degraded both types of polyesters (synthetic and natural polyesters).
The finding indicates the presence of at least two different extracellular enzymes for this strain, probably a PHB depolymerase and a lipase. Lipases are known to be able to hydrolyze synthetic polyesters such as PCL, while PHB depolymerases, which are enzymes designed by nature to cleave ester bonds in natural PHB, usually are not able to hydrolyze synthetic polyesters.
The results obtained in this work provide useful information about the degradation behavior of polyester materials under mesophilic conditions, which is of importance for instance for the application of biodegradable plastics in agriculture. It is obvious that a great number of organisms exist, which are able to degrade various polyesters even under mesophilic to psycryophilic conditions where here fungi play an important role. In contrast it was found in previous investigations that at higher temperatures (e.g. in compost) actinomycetes are the predominant polyester degrading organisms. The characteristics of a mesophilic extracellular enzyme isolated in this work leads to the supposition that as already shown for thermophilic conditions, lipase – like enzyme are responsible for the first step in the degradation procedure.

6. Zusammenfassung

Die Arbeit umfasst Untersuchungen zum Bioabbau von synthetischen Polyestern wie Poly(tetramethylen adipat – co – tetramethylen terephthalat) (BTA Copolyester), Poly(ε-caprolacton) (PCL) und Polyteramethylenadipat (SP 4/6) sowie des natürlichen Copolyesters Poly(hydroxybutyrat-co-hydroxyvalerat) (PHBV) unter mesophilen Bedingungen. Unter Verwendung einer "Clear-Zone" Methode mit Agar-Platten konnten insgesamt 47 polyesterabbauende Microorganismen-Stämme (15 Pilze, 24 Actinomyceten, 8 allgemeine Bakterien) aus reifem Kompost sowie einer Pflanzerde isoliert werden, wobei PCL und ein BTA-Copolyester als Selektionssubstrat verwendet wurden. Acht der am besten abbauenden Stämme wurden ausgewählt, identifiziert und für weitergehende definierte Abbauversuche verwendet. Die Bildung eines induzierbaren, extrazellulären, PCL-abbauenden Enzyms konnte für ein Isolat nachgewiesen werden. Die Hydrolase wurde bezüglich ihrer grundlegenden Eigenschaften charakterisiert.

Die polyesterabbauenden Mikrobenisolate konnten bezüglich ihrer Substratspezifität in zwei Gruppen unterteilt werden. In die erste Gruppe wurden Isolate eingeordnet, die nur in der Lage waren, die synthetischen Polyester, wie BTA-Copolyester, PCL oder SP4/6, abzubauen, aber nicht den natürlichen Polyester PHB angriffen. Zu dieser Gruppe gehörten Stämm der Pilze *Arthrobotrys amerospora* (EB1)*, Acremonium strictum* (EB14)*, Cladosporium herbarum* (EB2T) und *Gliocladium roseum* (G1). Die zweite Gruppe besteht aus Stämmen die sowohl synthetische als auch natürlichen Polyester depolymerisieren können. Hierzu gehören Stämme der Pilze *Fusarium solani* (EB10), *Aspergillus fumigatus* (EB19) und *Gliocladium roseum* (G2) sowie Stämme der Actinomyceten der Spezies *Microbispora rosea subsp. rosea* (Isolate E11 and Act3) und *Streptomyces thermocarboxydus.* Wie aus Ergebnissen in der Literatur zu erwarten war, wurden von den meisten Stämmen PCL – Filme schneller abgebaut als der Copolyester BTA 45:55. Der Stamm *Arthrobotrys amerospora* (EB1) hingegen hydrolysierte den aliphatisch-aromatischen Copolyester schneller als das aliphatische PCL. Dies deutet darauf hin, dass dieser Mikroorganismus offensichtlich eine Enzymsystem besitzt, das speziell für die Hydrolyse der aromatischen Polyester geeignet ist.

Mit 7 Pilzisolaten wurden dann vergleichende Abbautests bei verschiedenen Temperaturen (30°C, 25°C,15°C und 4°C) durchgeführt. Für die unterschiedlichen Isolate wurden dabei verschiedene Temperaturabhängigkeiten des Polyesterabbaus beobachtet. Während die meisten Isolate höchste Abauraten im oberen untersuchten Temperaturbereich aufwiesen, zeigten 3 Stämme (*Fusarium solani* (EB10), *Cladosporium herbarum* (EB2T) und

Acremonium strictum (EB14)) auch noch bei 4°C eine deutliches Abbaupotential gegenüber den untersuchten Polyestern.
Der Pilzstamm *Arthrobotrys amerospora* (EB1) wurde eingesetzt, um Abbauuntersuchungen mit dem Copolyester BTA 45:55 in Mineralsalzmedien durchzuführen. Hierbei konnten Pulver bzw. Nanopartikel des Copolyesters innerhalb von 12 Wochen zu 80% abgebaut werden.

Eine extrazelluläre, PCL- abbauende Aktivität konnte in einem Kulturüberstand des Stammes *Microbispora rosea subsp. rosea* nachgewiesen werden. Es wurde gezeigt, dass die Hydrolase durch die Gegenwart von PCL und BTA-Copolyester induziert wird, während bei Vorhandensein von weiteren Kohlenstoffquellen (Glukose, Komplexmedium) keine extrazelluläre Hydrolyseaktivität zu beobachten war. Die aufkonzentrierte Rohenzymlösung wies Hydrolyseaktivität gegenüber Triglyceriden (Triacetin und Triolein) sowie verschiedenen synthetischen Polyestern (SP 4/6, PCL, BTA Copolyester) auf, während der natürliche Copolyester PHBV nicht angegriffen wurde, obwohl der Mikroorganismus selber in der Lage war, beide Arten von Polyestern abzubauen.
Diese Beobachtung stützt die Vermutung, dass der Stamm zumindest zwei Enzymsysteme, vermutlich eine PHB-Depolymerase und eine Lipase, ausscheiden kann. Es ist bekannt, dass Lipasen in der Lage sind synthetische Polyester anzugreifen, während PHB-Depolymerasen, die von der Natur zur Depolymerisierung von Polyhydroxyalkanoaten vorgesehen sind, dies normalerweise nicht können.
Die in dieser Arbeit erhaltenen Ergebnisse geben wichtige Informationen bezüglich des Bioabbaus von Polyestern unter mesophilen Bedingungen. Dies ist insbesondere hinsichtlich von Anwendungen bioabbaubarer Polymere z.B. im Agrarbereich von Interesse. Offensichtlich existieren in der Natur eine große Anzahl von Mikroorganismen, die in der Lage sind, verschiedene Polyester auch bei niedrigen Temperaturen abzubauen, wobei hier Pilze eine besondere Rolle spielen. Im Gegensatz dazu sind unter thermophilen Bedingungen (z. B. bei der Kompostierung) Actinomyceten die hauptsächlichen Polyesterabbauer. Die Eigenschaften des in dieser Arbeit isolierten extrazellulären, polyesterabbauenden Enzyms legt die Vermutung nahe, dass hier, wie bereits für thermophile Bedingungen gezeigt, lipase-ähnliche Enzyme für der ersten Schritt im biologischen Polyesterabbau verantwortlich sind.

7. Materials and Methods

7.1. Polymers

The chemical structure of the different linear polyesters which were chosen for the degradation studies are summarized in Fig 7.1.

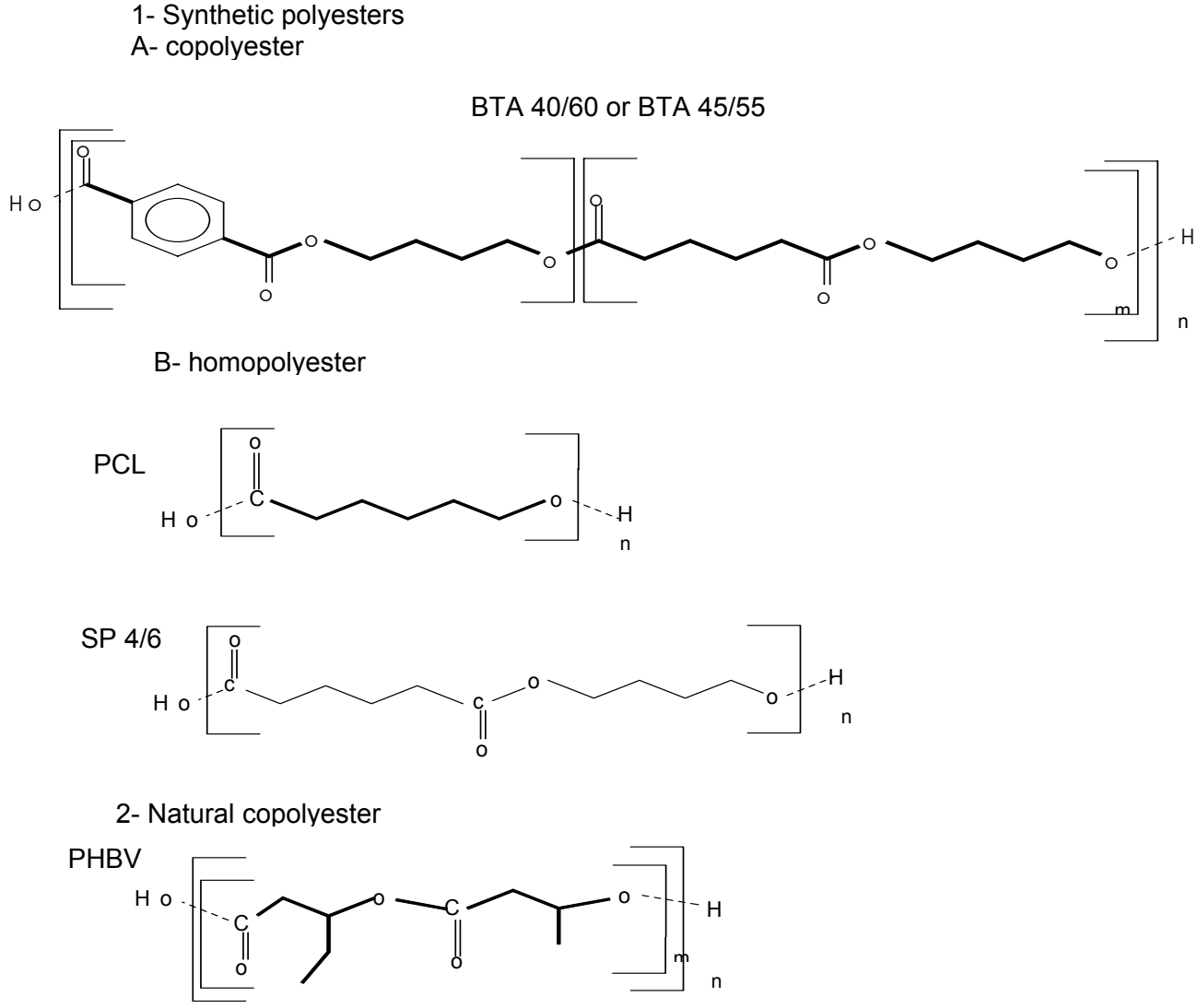


Fig. 7.1. Chemical structure and classification of the linear polyesters used during this study.

Table 7.1 summarizes the chemical components, the composition and the physical characteristics of the polyesters.

Table 7.1. Chemical structure, components, melting temperature and molar masses of the tested polymers.

Polymer	Component (s)	T_m (°C)[b]	M_w (g/mol)[c]	Source
BTA 40:60	1,4 butandiol/ adipic acid (60 mol %) / terephthalic acid (40 mol %)	99	47600	Hüls AG, Marl, Germany
BTA 45:55 (Ecoflex)	1,4 butandiol/ adipic acid (55 mol %) / terephthalic acid (45)	100	114000	BASF, Ludwigshafen, Germany
PCL	ε-caprolacton	60	50000	Polyscience, Inc. Warrington, USA
SP 4/6	1,4 butandiol/ adipic acid	62,3	40000	GBF, Braunschweig
PHBV	3-hydroxybutyrate/ 3-hydroxyvalerate (11,6 mol%)	180	397000	ICI, Bellingham, United Kingdom (as Biopol BX)

[b]T_m melting temperature; maximum of DSC melting peak.

[c]M_w weight average molar mass (determined by gel permeation chromatography, based on polystyrene calibration)

7.1.1. Polyester sample preparation for degradation tests

In order to work under defined and reproducible conditions, the polyesters were processed into thin films by compression molding using a hydraulic press (Ident.-Nr. 062566, perkin Elmer, Überlingen) with two thermostatable metal plates (PIN 15515, Specac, England) as described by Witt et al., (1995). The polymer powder was placed between two teflon coated paper sheets separated by an metal spacer (varying thickness were used to determine the film thickness required for each specific test). The polyester material (powder, thick pre-pressed films) was then compression molded for 2 minutes applying a pressure of 2 tones. The temperature was chosen 5°C below the melting temperature of the polyesters (see Table 7.1). BTA 45:55 (Ecoflex) and PCL were available as sheets (thickness: 60 and 75 µm, respectively). Using punches with defined diameters films of defined surface areas were cutted out.

Sterile circular films of the polyester 2.5 cm in diameter were used in the degradation tests on agar plates or in liquid medium. The surface area was 4.91 cm² when the films were

treated with the microorganisms in one surface but it was 9.82 cm² when the films were treated with the organism for the both surface film or in case of using the films in the degradation test in liquid medium.

7.1.2. Sample sterilization

The vaccum dried and pre-weighed films were sterilized by one of the following methods.

a- UV irradiation (Wallhäuser, 1984).

Each film surface of the polyester films was exposed for 15 min to UV-irradiation using an UV lampe (UVC 30; Hereaus, Holding GmbH, Hannover, Germany; 254 nm, 6W cm² with a distance of 20 cm). The films were placed on a irradiation area of 38 by 18 cm at a constant distance of 15 cm from the irradiation source.

b- Hydrogen peroxide treatment (Wallhäuser, 1984).

For degradation tests in liquid cultures the processed films were inserted singly in small petri dishes (∅= 35 mm, Greiner, Frieckenhausen) and exposed per each side to 10 % (vol/vol) H_2O_2 for 1 hour. After removing the H_2O_2-solution the films were dried at room temperature over night and washed thereafter in three subsequent volumes of 500 ml sterile distilled water using a sterile forceps.

c- Autoclaving

Optionally, the processed and pre-weighed PHBV films, having a melting temperature of 180°C, were sterilized in glass petri dishes at 121°C and a pressure of 1 bar for 20 minutes (Matachana, Zirbus, Osterode). If the polyester (PHBV) was used as powder for agar plate clear zone test PHBV powder was sterilized together with the medium constituents. Direct autoclaving of polyester films of the other materials were not possible due to their low melting points and the tendency to agglomerate (see 7.2.3).

7.2. Microbiological investigations

7.2.1. Source of organisms

Two different microbial sources were used for the isolation of the microorganisms.

1. Mature compost 6 months old (made from green waste (compost Plant Watenbütel, Watenbütel, Germany) and biofilms from BTA 45:55 (Ecoflex) films which were incubated in the compost at room temperature for 6 months.
2. Gardening soil (Deutsche Einheitserde Werke)

7.2.2. Media for cultivation and degradation experiments

The compositions of the media used in this work are listed in Table 6.2. Media sterilization was performed by autoclaving at 121°C and 1 bar pressure for 20 minutes (Matachana, Zirbus, Osterode).

The pH values of the media were adjusted prior to sterilization with 0.1 M sodium hydroxide or hydrochloric acid to the desired value.

Table 7.2. Cultivation media

Medium[a]	Composition (per 1L)
Mineral salt vitamín media (MSV)[b]	The following components were dissolved in demineralized water, Solution (a) KH_2PO_4 37.5 g, Na_2HPO_4 69.7 g (dissolved in 1000 ml) Solution (b): $MgSO_4.7H_2O$ 22.5 g (dissolved in 1000 ml) Solution (c): $CaCl_2$ 27.5 g (dissolved in 1000 ml). Solution (d): NH_4Cl 20 g (dissolved in 1000 ml). Solution (e): $FeSO_4.4H_2O$ 2 g, $ZnCl_2$ 70m g, $MnCl_2.2H_2O$ 100 m g, H_3BO_3 6m g, CoCl2.6H2O 190 mg, $CuCl_2.2H_2O$3 mg, $NiCl_2.6H_2O$ 240 mg, $Na_2MoO_4.2H_2O$ 36 mg, $Na_2WO_4.2H_2O$ 33 mg, $Na_2SeO_3.5H_2O$ 26 mg dissolved in 10 ml of HCl (25% 7.7 mol/L) and added to 1000 ml with water. Solution (f): biotine 0.6 mg, niacinamide 2 mg, *p*-aminobenzoate 2 mg, panthotenic acid 1 mg, pyridoxal hydrochloride 10 mg, vitamin B12 5 mg, folic acid 2 mg, riboflavin 5 mg, DL-6,8-thioctic acid 5 mg, thiamin dichloride 1 mg dissolved in 100 ml of H_2O. The vitamine solution was membrane-filtered (pore size 0.2 µm). the final medium was prepared by mixing 100 ml of solution a with 1 ml of solution b, c, e, and 10 ml of solution d, then made up to 1000 ml with demineralized water).
Complex media[c]	
Maltextract medium[d]	maltose/technical 12.75 g, dextrin 2.75 g, bacto glycerol 2.35 g, Bacto Pepton 0.78 g,1000ml demineralized water, (or 33.6 g/1000ml demineralized water, pH 4.7 Difco (0112-17)).

Potato Dextrose [d] (PDA)	infusion from potatoes (see blow), 1000 ml demineralized water, glucose 20 g, potato infusion, (boil 200 g scrubbed and sliced potatoes in 1000 ml water for 1hour. Pass through fine sieve. Avoid new potatoes.)
Trypticase soy broth (TSB)[e]	Trypticase soy broth 30 g, 1000 ml demineralized water (or: peptone from casein 17 g, peptone from soymeal 3 g, D(+) glucose 2,5 g, NaCl 5 g. K_2HPO_4 2,5 g, demineralized water 1000 ml
GYM Streptomyces Medium[e]	Glucose 4 g, yeast extract 4 g, malt extract. 10 g, $CaCO_3$ 2g,1000 ml demineralized water,
LB (Luria- Bertani) medium[e]	Trypton 10 g, yeast extract 5g, NaCl 10 g, demineralized water 1000 ml.
Standard count agar (Merck)	suspend 25 g/L of standard count agar powder in 1000 demineralized water by heating in a boiling water bath; pH 7.2 ± 0.2; autoclave 20min at 121°C.

[a] 20 g agar l^{-1} was added to all media; pH was adjusted to 7.0 ± 0.2; media were autoclaved for 20 minutes at 121°C.

[b] mineral salt vitamin medium used for the enrichment, the screening and biodegradation tests for the microorganisms.

[c] complex media used for purification and cultivation of isolated strains, while ([d]) were used for the fungal isolates, and ([e]) were used for the bacterial isolates.

7.2.3. Preparation of clear zone plates

a. Clear zone plates with synthetic polyesters (BTA 40:60, BTA 45:55 (Ecoflex), PCL and SP4/6 agar plates)

1. For synthetic polyesters a suitable method to prepare turbid agar plates was described by (Abou Zeid et al., 2001). The polyester (0.25 g) was dissolved in 5 ml methylene chloride and the solution was then emulsified by sonication into 250 ml of MSV medium containing 1.5% (w/v^{-1}) agar-agar. The emulsion was then stirred continuously while heating for at least 30 minutes to evaporate the solvent completely. The pH was adjusted to pH 7±0.2. The autoclaved medium resulted in homogenous opaque plates where it was poured into plates (9 cm in diameter) with 15 ml/plate.

2. 1000 mg from of synthetic polyesters were dissolved in 50 ml tetrahydrofuran, filled up to 100 ml with aceton, and finally filled up to 200 ml with deonisd water. Polymer flakes were

filtered off through a sieve (pore size 100 µm). The organic solvent was then removed with a rotational evaporator from the aqueous solution. Then 2x1 and 2x5 ml from the prepared suspension were dried to estimate the final concentration of the suspension.
The sterile mineral salt medium (MSV) with 20 g/l agar was mixed with concentration 0.1% from polymer suspensions (from b) before cooling the agar (at 50°C). 15 ml were poured for each plate (9 cm in diameter), homogenously turbid plates were obtained.

b. Clear zone plates with natural copolyester (PHBV)

For clear zone tests (Augusta et al., 1993). PHBV powder was added to MSV medium at a final concentration of 0.1% (w/v) and the mixture was ultra-sonicated for five to seven minutes at 90 duty cycles using a branson sonifier (Branson Ultrasonic Cooperation, Danburry, CT, USA). The medium was sterilized at 121°C and a pressure of 1 bar for 20 minutes (Matachana, Zirbus, Osterode) because it have melting point 180°C. 15 ml medium were poured before cooling for each plate (9 cm in diameter)

7.3. Screening and isolation procedures of polyester-degrading microorganisms

7.3.1. Screening

A two - step procedure turned out to be the appropriate strategy for the isolation of polyester degrading microorganisms. These were as follow:

1. preparation of inocula

A three - step procedure were used for the preparation of the inocula. These were taken by either:

●Direct from compost

Dissolving 10 g of mature compost in 90 ml sterilized mineral salt vitamin liquid medium in conical flask (250 ml) (Kleeberg et al., 1998). After thoroughly shaking at 100 rpm and room temperature for 10 min, the dilution series were prepared in tube glass containing 9 ml MSV liquid medium up to $1:10^5$ with sterilized mineral salt vitamin liquid medium were prepared. The three later dilutions were used to inoculate plates containing dispersal mineral salt vitamin agar medium with PCL (0.1% w/vol) by spreading 100 µl of suspension on agar plates.

● From preadapted biofilms grown on BTA 45:55 (Ecoflex) films

1. Scraping off preadapted biofilms grown on BTA 45:55 films which were incubated in compost reactors at room temperature for 6 months (Kleeberg et al., 1998). The biofilm was

suspended in 1ml of sterilized mineral salt vitamin liquid and used for the inoculation of the different polyester-agar plates.

● **From gardening soil**

Enrichment cultures of microorganisms capable of degrading the polyester were established from gardening soil. 10 g of soil were suspended in 90 ml MSV – medium. The medium was supplemented with 0.1% w/v of suspension from polyesters (BTA 45:55 (Ecoflex) or PCL) to abbreviate the incubation time. Cultures were incubated at 30°C and 160 rpm for 6 weeks. After a week, 10 ml of culture broths were transferred into fresh 90 ml mineral salt liquid medium containing 0.1% w/v of suspension from BTA 45:55 (Ecoflex) or PCL. This procedure was repeated six times. Dilutions series were prepared after the final time (after 6 weeks) from enrichment culture in tube glass containing 9 ml MSV liquid medium up to $1:10^6$.100 µl from the three later dilution from series were spreaded on emulsified MSV-agar containing BTA 45:55 (Ecoflex) or PCL (0.1%w/vol) by using Drigalisky triangle.

2. Incubation of the polyester – agar plates for different inocula

MSV-agar plates containing BTA copolyester (BTA 45:55 (Ecoflex) or BTA 40:60 (0.1 % w/vol) or PCL (0.1%w/vol) were inoculated with 100 µl from the prepared inocula by spreading it on the surface of the agar plates with a sterilized Drigalisky glass triangle. The plates were sealed in polyethylene bags and were incubated at room temperature or at 30°C for 1 week, respectively. The plates were examined daily for growth of colonies and clear zone formation around the colonies

7.3.2. Purification of polyester depolymerizing strains

Positive strains forming colonies surrounded by clear zones after incubation for 7 days at room temperature (24-26°C) and 30°C, respectively were isolated by picking the colonies using sterile tooth picks or needle inoculation and were further purified on complex media (Potato Dextrose agar and malt extract agar for fungal isolates, GYM and TSB for actinomycetes and LB medium for general bacteria) (see 7.2.2). For bacterial strains the standard spatial streaking method on solid agar plates was used. Fungal isolates were purified by using acidic complex medium such as malt extract agar (pH 4.7) or by addition of ampecillin 800 mg/l to complex agar media (Potato Dextrose Agar).

7.3.3. Preservation of isolates

a. Fungal isolates

The fungal isolates were maintained on complex agar plates or MSV-agar slants containing BTA-copolyester or PCL (0.1% w/v) as a sole carbon source at 4°C. For recultivation, the fungal growth was suspended in liquid complex media and was cultivated on complex agar med or by cultivation of disc (5 in diameter) which bear the fungal mycelium on complex medium.

b. Bacterial isolates

Bacterial isolates were preserved in 50% (v v^{-1}) glycerol (87%) at –20°C. For recultivation, 1 loopful of each glycerol suspension was streaked on GYM or LB media (GYM for actinomycetes and LB for general bacteria). After 3-4 days of incubation on the complex media single colonies were picked and were inoculated in 30 ml of the same liquid complex medium. This suspension was used as inoculm for the degradation tests.

7.4. Identification of the selected isolates

7.4.1. Fungal isolates

Fungal isolates were identified according to cultural and morphological characteristics in German Culture Collection in Braunschweig, (DSMZ), Germany.

7.4.2. Bacterial strains

The identification of the bacterial isolates was performed in German Culture Collection in Braunschweig, (DSMZ), Germany. The bacterial isolates (E11, Act3, and Act23) were identified according to morphological (Hütter, 1967), physiological, chemotaxonomical (Fatty acid) investigation (Bergey's 1987, and Kämpfer et al., 1991) as well as the molecular biology (phylogenetic) which based on partial sequences analysis for 16S rDNA gen (Maidak et al. 1996; Rainey et al. 1996).

● Extraction and analysis of fatty acid

For classification or identification of bacteria the presence of distinct fatty acids and their relative amount is analyzed and compared with the fatty acid profiles of reference strains (Busse et al.,1996). Fatty acids methyl esters were obtained from wet biomass (ca. 40 mg) by saponification, methylation, and extraction (Kuykendall et al., 1988). The fatty acid methyl ester mixtures were separated by using a model 5898A microbial identification system apparatus (Microbial ID, Newark, Del.). Peaks were automatically integrated and fatty acids were identified by the Microbial Identification System Standard Software (Microbial ID).

● DNA base composition and 16 rDNA partial sequence analysis

For 16S ribosomal DNA (rDNA) sequencing, the genomic DNAs of E11, Act3, and Act23 were extracted and the 16S rDNA partial sequence analysis is based on the determination of

parts of the 16S rDNA nucleotide sequence via direct sequencing of the 16S rDNA and amplifying each part of the genomic DNA using PCR. For the extraction of the genomic DNA, the PCR amplification of the rDNA and the purification of the PCR products the methods described by Rainey et al., (1996) were used. Purified PCR products were sequenced with Taq Dye deoxy terminator cycle sequencing kits (Applied Biosystems, Weiterstadt, Germany) as directed in the manufacture's protocol. The resulting sequence data were interpreted according to and compared with the 16S rDNA nucleotide sequences of representative organisms of the main bacterial lineages available from public databases Maidak et al., (1996).

7.5. Microscopic examinations

For purification examination and morphological investigations samples the different strains were studied using the phase contrast light microscope (Axioscop, Zeiss, oberkochen, Germany). The macroscopic appearance and culture morphology on solid agar media as well as polyester film surfaces were studied using a Stemi 2000 microscope (Zeiss, Jena, Germany).

7.6. Degradation test with isolated strains

7.6.1. Polyester depolymerization measured by clear zone formation

The potential of the isolates to depolymerize different polyesters was preliminary examined using the clear zone method (Augusta et al., 1993)

For the fungal isolates each agar plate containing the different polyesters was inoculated in the center with a disk (5 mm in diameter) from a complex medium (Potato Dextrose agar) with the fungal mycelium of a culture grown for 7 days at room temperature or 30°C. The turbid agar plates were incubated at room temperature and 30°C, respectively.

For the bacterial strains the turbid MSV-agar plates containing the polyester (0.1% w/v) were inoculated with bacterial colonies from agar plates (GYM for actinomycetes and LB for general bacteria) cultures of the different strains with inoculation needles and incubated for 1 week at 30°C or from liquid cultures by spreading of the cell suspension (100 µl) by using a glass triangle. The plates were incubated for 1 week at 30°C.

The increase in clear zone diameters developing on the MSV-agar plates was followed up periodically and measured by slide gauge. Plates not inoculated with fungi were used as control. Experiments were made in tri-plicates.

7.6.2. Weight loss determination of polymer films on agar plates

As an quantitative measure for polyester depolymerization the determination of the weight loss of polymer films laid on agar plates and inoculated with the purified isolates

Three preweighted, sterile circular films (25 mm diameter, surface area assessible for degradation (one side for fungal degradation = 4.91 cm², both sides for bacterial degradation = 9.82 cm^2) of the synthetic and natural polyesters were placed on a MSV- agar plate and inoculated with the different strains.

For the fungal strains inoculation was done by placing a 5 mm disc of agar-plate culture of the fungus (Potato dextrose agar medium, incubated for 7-20 days at 30°C) in the center of the polyester film. For the degradation tests with actinomycetes and general bacteria the polymer films were inoculated with 200 µl of a seed culture (10^7 CFU/ml). The seed culture was prepared in GYM-medium for actinomycetes and in LB-medium for general bacteria (30 ml medium in a 100 ml flask shaked at 160 rpm and 30°C for 3-4 days) (4 days for actinomycetes and 3 days for general bacteria).

The degradation times on the agar plates was varied according to the incubation temperature (4 weeks for 30°C, 6 weeks for 15°C and 25°C, 32 weeks for 4°C).

Sterile controls incubated over the same period of time were performed and showed no weight loss due to a abiotic hydrolysis of the polyester samples.

7.7. Biodegradation of BTA 45:55 (Ecoflex) with strain EB1

7.7.1. BTA 45:55 (Ecoflex) powder (0.1 - 0.2 mm Ø) at 30°C

100 mg (500 mg) of BTA 45:55 (Ecoflex) powder (0.1–0.2 mm Ø) were added to flasks (500ml and 1000 ml, respectively) containing 100 ml (500 ml) sterile MSV-medium (pH7). The tests were inoculated with a conidia suspension of strain EB1 (10^5 CFU/ml; 1 ml inoculm per 100 ml MSV-medium). The flasks were shaken at 160 rpm and 30°C for 4, 8 and 12 weeks. After termination of the incubation the medium was centrifuged at 10000 rpm for 30 min. The pellets were washed with 30 ml demineralized water two times and centrifuged as described before. The pellets were dried at 30°C to constant dry weight. The dry pellets were analyzed by GPC with regard to the polymer content (peak area).

6.7.2. BTA 45:55 (Ecoflex) nanoparticles (220nm) at room temperature

200 ml MSV-medium (pH7 $\pm$ 0.2) was supplemented with BTA 45:55 (Ecoflex) nanoparticels (0.1% w/v polymer) in 500 ml flasks. Each flask was inoculated with 16 discs (5 mm in diameter from potato dextrose agar medium with the mycelium of strain EB1) and were shaken at 160 rpm for 7 days at room temperature. The medium was then centrifuged at 10000 rpm for 20 min. The pellets were washed twice with 20 ml demineralized water and also centrifuged at 10000 rpm for 20 min and were dried at 30°C to constant dry weight. The

dry pellets were analyzed by GPC via peak area analysis with regard to the residual polymer content.

7.8. Optimization of the cultivation conditions

7.8.1. Fungal isolates

To optimize the conditions for the cultivation of the different isolates glucose was used as carbon source instead of polyesters. To find the appropriate glucose concentration for the following experiments, 30 ml mineral salt medium (MSV, pH 7) in 100 ml flasks was supplemented with different concentration of glucose (5, 10, 15 and 20 g/L). The medium was inoculated with 1 disc (5 mm in diameter) from an agar plate with the fungal mycelium (potato dextrose agar medium) where the particular strains have been incubated for 7 days at 30°C for EB1, EB10, EB14, EB19, G1 and G2 and at 15°C for strain EB2T. The inoculated flasks were then incubated at 30°C for the isolates EB1, EB10, EB14, EB19, G1 and G2 and at 15°C for strain EB2T for 1 week. The biomass was determined as the mycelial dry weight (g/30mL) by filtering the content of culture broth through a pre-dried and pre-weighed filter paper (5951/2 Schleicher & Scheuel, Dassel, Germany) and drying it at 60°C till a constant weight was achieved. All results reported are the means of triplicates sample measurement.

● Optimization of pH

30 ml mineral salt medium (MSV) was supplemented with 20 g glucose/liter in 100 ml flasks at pH 4, 5, 6, 7, 8 at 30°C for the isolates EB1, EB10, EB14, EB19, G1 and G2 and at 15°C for strain EB2T for 7 days. The inoculation and the mycelia dry weight determination was performed as mentioned above.

● Optimization of temperature

30 ml mineral salt medium (MSV) pH7 was supplemented with 20 g glucose/liter in 100 ml flasks. The flasks were incubated at 4, 15, 25, 30, and 40°C for 7 days. The inoculation and the mycelia dry weight determination was performed as mentioned above.

7.8.2. Bacterial isolates

For estimating the optimal glucose concentration for the following tests, 30 ml mineral salt vitamin (MSV) in 100 ml flasks were supplemented with different concentration of glucose (2, 4, 6, and 8 g/L) and inoculated with 1ml from a bacterial cell suspension (10^5 to 10^7 CFU/ml), from complex liquid GYM- medium for actinomycetes, and LB medium for general bacteria. The flasks were shaken at 160 rpm and 30°C for (4 days for actinomycetes and 3 days for general bacteria). When the protein content of cells in liquid media had to be determined, the bacterial cells were first digested and afterwards the protein content was determined. For this purpose, 1mL from the culture was centrifuged at 14000 rpm for 10 min,

and the pellets were resuspend and washed three times with 80 mM phosphate buffer pH 7.1 and centrifuged again. The pellets were then suspended in 1 ml 1 N NaOH and incubated for 2 hour at 60°C in a thermo-mixer (5436, Eppendorf, Hamburg). The mixture was then centrifuged and the supernatant was used for protein determination as described by Lowry et al., (1951).

● Optimization of pH

The experiments were carried out in 30 ml MSV liquid medium with 8 g glucose / liter at pH 6, 7, and 8 at 30°C for 3-4 days (4 days for actinomycetes and 3 days for general bacteria) in 100 ml flasks. Inoculation and biomass determination was performed as described above.

● Optimization of temperature

30 ml MSV liquid medium pH 7 was supplemented with 8 g glucose and incubated at 25, 30, and 40°C for 3-4 days (4 days for actinomycetes and 3 days for general bacteria) Inoculation and biomass determination was performed as described above.

7.9. Analytical methods

7.9.1. Gel permeation chromatography

The molecular weight distribution of the polymeric materials was determined using a Techlab chromatograph equipped with a pre-column (Pl gel®, 5 guard, 50 × 7.8 mm, Latek, Eppenheim, Germany) and a separation column (Pl gel®, 300 × 7.5 mm (5×10^4 or 10^3), Latek, Eppenheim, Germany) with a functional molecular weight range of 5000–6000 g mol^{-1} or 11800-500000 g mol^{-1}. Aliquots of 100 µl of the sample (BTA 45:55 (Ecoflex) or PCL) under investigation were previously dissolved in chloroform and injected. Chloroform (HPLC grade, degassed and filtered) was used as the mobile phase at a flow rate of 1.0 ml min^{-1} at room temperature. The GPC unit is equipped with a refractive index (RI) detector (SKD Shodex RI SE-51) and a UV-detector (Gynkotek SP-6V). Number-average (M_n) and weight-average (Mw) molecular weights were calculated relative to polystrol standards (Software: Chromostar, Bruker). Calibration curves of pure BTA 45:55 (Ecoflex) and PCL were established in a concentration range of 0.5 – 2.5 mg^{-1} in chloroform. The concentration of polyesters were calculated by GPC via peak area analysis with regard to the residual polymer content.

7.10. Cultivation of strain E11 in liquid medium for Enzyme production test

a. Growth of strain E11 on PCL films

The experiments were carried out to study of production of the enzyme from strain E11 in mineral salt vitamin liquid medium.

Sterile circular polyester film (25 mm in diameter, surface are 9.82 cm², 38.4 – 42.6 mg) were added to 50 ml MSV liquid medium in 250 ml flasks and inoculated with 3 ml of a cell suspension of E11 (GYM liquid medium, 10^7 CFU/ml; incubated at 30°C and 160 rpm for 4 days). The flasks were shaken at 160 rpm and 30°C. The degradation of films was examined after incubation for the desired incubation period time (1 week) by weight loss determination. The growth and activity of the enzyme was determined daily. The growth was determined by determination of intercellular protein (see 7.11 a). The activity of the enzyme was determined daily by filtration of the supernatant through (0.2 µm, Minisart, sartorius, Göttingen, Germany) and it was determined by decreasing in the turbidity of PCL suspension as shown in (7.12.1). In the large scale, the cultivation broth was centrifuged at 11400 rpm for 20 min and was then filtrated (RC 58 membrane filter, 0.2 µm, 47mm Schleicher und Schüll, Dassel, Germany) and concentrated by ultrafiltration process. The cultivations were compared with non-inoculated controls.

b. Effect of different carbon source on growth strain E11 and enzyme induction

The experiments were carried out to study different carbon source on the induction of the enzyme from strain E11 in mineral salt vitamin liquid medium.

● On mineral salt vitamin liquid with PCL nanoparticles, PCL+glucose (8 g/l) and BTA 45:55 (Ecoflex) nanoparticles.

50 ml of MSV medium (pH7 ± 0.2) was supplemented with PCL-nanoparticles (0.1% w/v), glucose (8 g/l), PCL+glucose and BTA 45:55 (Ecoflex) (0.1%) as sole of carbon source (250 ml flask).

● On GYM liquid with PCL nanoparticles

50 ml of GYM medium (pH7 ± 0.2) was supplemented with PCL-nanoparticles (0.1% w/v). Each flask for the media was inoculated with 3 ml of a cell suspension of E11 (GYM liquid medium 10^7 CFU/ml; incubated at 30°C and 160 rpm for 4 days) and were incubated at 160 rpm and 30°C for 6 days.

The growth and activity of the enzyme was determined daily. The growth was determined by determination of intercellular protein (see 7.11 a) The activity of the enzyme was determined daily by filtration of the supernatant through by decreasing in the turbidity of PCL suspension (OD_{650nm}) (0.2 µm, Minisart, sartorius, Göttingen, Germany) and it was tested as shown in (7.12 1).

One experiment was carried out by inoculation of 30 ml of GYM medium in 100 ml flasks by 1 ml of a cell suspension of E11 (GYM liquid medium 10^7 CFU/ml; incubated at 30°C and 160 rpm for 4 days) and were incubated at 160 rpm and 30°C for 6 days. The growth was determined by determination of intercellular protein (see 7.11 a).

7.11. Determination of protein content

The protein concentration of cells and enzyme samples were determined according to the method by Lowry et al., (1951) using bovine serum albumin as standard protein.

a-Determination of the intracellular protein content

When the protein content of cells in liquid media had to be determined, the bacterial cells were first digested and afterwards the protein content was determined. For this purpose, 1mL from the culture was centrifuged at 14000 rpm for 10 min, and the pellets were resuspend and washed three times with 80 mM phosphate buffer pH 7.1 and centrifuged again. The pellets were then suspended in 1 ml 1 N NaOH and incubated for 2 hour at 60°C in a thermo-mixer (5436, Eppendorf, Hamburg). The mixture was then centrifuged and the supernatant was used for protein determination as described by Lowry et al., (1951).

7.12. Measuring enzyme activity

1. By the decrease of the optical density of a PCL-suspension

The test principle is based on the decrease of the optical density (OD_{650nm}) of PCL-particles during the enzymatic degradation (Jendrossek et al., 1993A; Müller and Jendrossek, 1993; Murphy et al., 1996; Tomasi et al., 1996; Oda et al., 1997; Oda et al., 1997 Abou-Zeid et al., 2001). The standard assay mixture contained 0.25 ml of 20 mM phosphate buffer (pH 7), 2 ml of PCL suspension (38 µg/ml) and 0.25 ml of sterile crude enzyme in a total volume of 2.5 ml. After determination of the initial OD_{650nm} of this mixture, it is incubated at 30°C. The decrease of the optical density of this mixture allows the tracing of the enzyme activity. The enzyme activity is calculated by linear regression from the slope of the decrease in turbidity during a period of time of 5-60 min (5 min for the concentrated crude enzyme after ultrafiltration process and after purification process and 60 min for supernatant (non concentrated), where one activity unit (U) is defined as an decrease in turbidity of the PCL suspension of 0.001 OD per minute.

2. Measuring of PCL- hydrolase activity towards triglyceride (Triolein + Triacetin) by titration method

The method is based on a continuous neutralization (pH-stat titration) of the acid groups liberated from the ester cleavage of the triglyceride chains, due to the enzyme activity, with 0.1 M NaOH. The volume of base consumed is plotted against the incubation time of the test. The reactor consists of a small 10 ml-glass vials, externally thermostated with a water bath at 30°C. The reactors were closed with rubber stopper through which, a small tube for the addition of base was inserted. The system was flushed with N_2 to prevent the penetration

and dissolution CO_2, in the reaction mixture. The pH-meter was calibrated at 30°C with buffer pH4, pH7, and pH9. The end-pH of the reaction was fixed at 7.

The emulsion solution was prepared by dissolving 4.475 g NaCl, 0.103 g KH_2PO_4, 190 g glycerin (87%) and 1.5 g gum arabicum in 250 ml of deioinzed water; the mixture was stirred for 2 h. 1 ml of the triglyceride mixture (0.3 ml triolein and 0.7 ml triacetin) was added to 4 ml of the emulsion solution and 3.6 ml of deioinzed water and the mixture was emulsified by Ultraturrax stirring (13500 rpm) for 1 minute. 5,5 ml of this emulsion was filled into the titration reactor, the pH was adjusted to pH 7 and the temperature to 30°C. After that, 500 µl of concentrated crude PCL-hydrolase (1.96 mg/ml) was added and the titration was started The activity of the enzyme was calculated by linear regression from the slope of NaOH addition between 5 and 45 min of the test.

3. Substrate spectrum of crud enzyme different polyesters via clear zone formation

The culture broth was obtained by culturing of E11 strain with PCL films One sterile circular polyester film (25 mm in diameter, 39.7-42.9 mg and with surface area 9.82 cm²) was added to 50 ml MSV liquid medium in 250 ml flasks and inoculated with 3 ml of a cell suspension of E11 (GYM liquid medium 10^7 CFU/ml; incubated at 30°C and 160 rpm for 4 days) The flasks were shaken at 160 rpm and 30°C for 3 days.

After fragmentation the films to small fragments, the culture broth was centrifuged at 11400 rpm for 20 min and the supernatant was then filtrated through a sterile membrane filter (RC 58 membrane filter, 0.2 µm, 47 mm, Schleicher and Schüll, Dassel, Germany). The supernatant was concentrated (by ultrafiteration with 10 KDa cut off membrane, see 6.9.6.1) 100 µl from the sterile and concentrated crude enzyme (with concentration of protein 0.94 mg/ml) was put in punched out holes (diameter 5 mm) in MSV-agar supplemented with 0.1% w/v of polyester suspensions (BTA 45:55, BTA 40:60, PCL, SP 4/6 and PHBV). The plates were incubated at 30°C for 15 h. Diameter of clear zone were measured by a slide gauge.

7.13. Enzyme purification

1. Ultrafiltration

The ultrafilteration was carried out using an Omega™ unit (Pall- Gelman Sciences, Dreich, Germany) with a polysulfone membrane with a cutt off of 10 KDa and a surface area of the membrane 23.2 cm². The ultrafilteration process was carried out with N_2 gas at 3 bar pressure in a cooling room at 4°C. After usage, the ultrafilteration membrane was washed with 0.1 N NaOH and distilled water and stored at 4°C in a 10 % (v v^{-1}) ethanol solution.

2. Dialysis

Dialysis was carried out for 12 – 24 hours at 4°C in dialysis tubing's (Spectra/PorR MWCO: 6000 – 8000, diameter 2.55 cm, Spectrum Medical Industries, INC., Laguna Hills, CA, USA) which were closed at both ends with clamps using. The tubes were placed in a 100 fold volume of the respective buffer. The buffer was changed every 4 – 8 hours.

3. Fast protein chromatography (FPLC)

Trials to purify the a target enzyme via ion exchange chromatography (Mono Q column 9315048; Pharmacia Biotech, Freiburg, Germany, was performed using a standard FPLC unit (LCC-500 Plus) equipped with automatic equilibration, injection and elution facilities (Pharmacia, Uppsala, Sweden) at room temperature. The Mono Q column was washed with 50 ml from 20 mM Tris/HCl-buffer pH9. 2 ml from the concentrated supernatant (protein concentration 1.42 mg/ml) was loaded onto a Mono Q column (9315048 Pharmacia), which was equilibrated with 13 ml from 20 mM Tris/HCl-buffer pH9. Afterwards the column was washed by with a linear gradient of 1mM NaCL in start buffer pH9 at flow rate of 0.3 ml/min (in total volume 40 ml).

Also the Vivapure Q-column, Vivascience, Hannover, Germany) as ion exchange chromatography was used in the purification of PCL hydrolase after Mono Q column according to the protocole from Vivascience company which is in Table 7.3.

Table 7.3. Protocol of Q column (as anionon exchange) for purification PCL- hydrolase after (Vivascience company)

Step	Procedure	Speed Mini L	Speed Min H	Time
Equilibration	Load 400 µl 20 mM Tris/HCl-pH9	500 g	2000 g	5 min
Sample loading	Load up to 400 µl sample	500 g	2000 g	5 min
1st wash step	Wash spin column with 400 µl 20 mM Tris/HCl- pH9	500 g	2000 g	5 min
2nd wash Step	Wash spin column with 400 µl 20 mM Tris/HCl- pH9	500 g	2000 g	5 min
Elution	Load up to 400 µl 1M NaCl in start buffer	500 g	2000 g	5 min

Buffers were filtered (RC 58 membrane filter, 0.2 µm, 47 mm, Schleicher and Schüll, Dassel, Germany) and degassed (30 min under continues stirring with a membrane vacuum pump

equipped with a vacuum controller, (Vaccumbrand, Wertheim, Germany) prior to the application to the FPLC unit to prevent air bubble formation in the columns.

7.14. Preparation of buffers

Table 7.4. lists the different buffer systems used throughout the present work and their applications after Kleeberg et al., (1999).

Table 7.4. Buffer system and their application

Buffer	pH-range	Molarity	Application
Phosphate buffer:			
KH_2PO_4/Na_2HPO_4	7.1± 0,1	80 mM	Wash the biomass
	7 .0± 0,1	20 mM	Enzyme purification (phenyl sepharose), Enzyme test
	5.5-8.0	20 and 100 mM	Determination of pH optimum of the PCL hydrolase
Citrate buffer:			
Citric acid/Na_2HPO_4	4.0 – 7.0	150 mM	Determination of pH optimum of the PCL hydrolase
Tris/HCl	9.1 ± 0.1		Enzyme purification (Mono Q and Q)

7.15. Analytical SDS gel electrophoresis

a. Sample preparation

The concentrated protein samples were diluted to a ratio 1:2 with sample buffer (Table 7.5) and heated at 95°C for 6 minutes in a thermo-mixer (5436, Eppendorf, Hamburg). Samples treated by this way can be stored at –20°C.

b. SDS-PAGE

The gel compositions and layer thickness are presented in Table 7.6. Ammonium persulfate (APS) and TEMED were added to the gel solutions directly before polymerization. The solution of the running gel was poured and direct overlaying the gel with ethanol (200 µl) to give a flat gel surface. After the polymerization (approx. 30 min) the water was removed. The solution of the stacking gel was poured the comb was inserted in the liquid stacking gel to obtain the gel well.

15 µl sample volume was injected to the well on the gel. Separation of proteins was performed at room temperature. Electrophoresis was performed with 50 V up to the entrance of the samples into the separation gel and increased afterwards to 150 V. The run was terminated, as soon as the run central front reached the end of the gel. Optionally, proteins were separated by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis by the method described by Laemmli (1970) using a Biometra Multigel long Electrophoretic unit (Biometra, Göttingen, Germany) and an electrophoresis power supply (EPS 601, Pharmacia Biotech, Freiburg, Germany) see Table 7.5 and 7.6 for the composition of the electrophoretic buffers and gels.

Table 7.5 Composition of the buffers required for SDS-PAGE

Sample buffer	**Electrophoretic Buffer**	**4x Lower Tris**	**4x Upper Tris**
100 mM Tris HCL, pH 6.8 200 mM DTT freshly prepared 4 % SDS 0.2 % Bromophenol blue 20 % Glycerin	30.3 g/L Tris base 144.0 g/L Glycin 10 g/L SDS	181.7 g/L base 4 g/L SDS pH 8.8 with HCL	60.55 Tris base 4 g/L SDS pH 6.8 with HCL

Table 7.6 Composition of the running gels for SDS-PAGE (gel thickness 1 mm)

Solution	**Running gel final concentration**	
	4 %	**15 %**
30 % Acryl amide / 0.8 % N, N-Methylenbisacrylamide	4.6 ml	49.5 ml
4 x Upper Tris	8.25 ml	-
4 x Lower Tris	-	25.1 ml
ddH2O	19.8 ml	23.8 ml
APS 10 %	79.2 µl	119 µl
TEMED	39.6 µl	79 µl

Proteins from Precision protein standard (Bio Rad, catalog 161-0362) were used as standards. After electrophoresis, proteins were stained with silver according to Merrill (1984).

7.16. Native gel electrophoresis

Under native conditions, separation of proteins depends on many factors including size, shape and native charge. One straight foreword approach to native gel electrophoresis is to leave out the SDS and the reducing agent (DTT) from the standard Laemmli SDS PAGE protocol. Thus, the sample buffer contains neither SDS or DTT (see Table 7.7 and 7.8), samples are not heated and the gel and electrode solutions are prepared without SDS (Hames 1990).

Table 7.7 Composition of the native sample and running buffers

Native sample buffer	**Running gel buffer**
62.5 mM Tris, pH 6.8	25 mM Tris, pH 8.6
30 % (v/v) Glycerin (100%)	192 mM Glycin
0.25 g l^{-1} Bromophenol blue	prepared as 10 fold stock buffer, 4°C.

Table 7.8 Composition of the stacking and running gels for native gel electrophoresis (gel thickness 1 mm)

Solution	**Stacking gel**	**Running gel**
ddH2O	3.2 ml	7.2 ml
Tris, 1.5 M, pH 8.8	-	3.8 ml
Tris, 0.5 M, pH 6.8	1.25 ml	-
Bis/acrylamide solution 30 % Acrylamide / 0.8 % N,N- Methylenbisacrylamide	0.5 ml	4 ml
TEMED	15 µl	20 µl
APS, 10 %	60 µl	120 µl

After electrophoresis, proteins were comasie blue stained.

In order to proof the activity of the purified PCL hydrolase the active enzyme sample was diluted on a ratio of 1:2 with the native sample buffer and applied to the gels. After running

two identical native gels with an active enzyme sample one whole gel was stained (commasie blue) to determine the position of the separated protein bands.

7.17. Lyophilization

Freeze-drying of solutions were done in a BETA-A lyophilizer (Christ, Osterode, Germany) at room temperature and 0.05 bar for 4 -12 days. The solutions were dried in a Epsilon 2-65D lyophilizer (Christ, Osterode, Germany).

7.18. Distribution of BTA 45:55 (Ecoflex) and PCL degrading microorganisms in different soils.

8 soil samples were collected from different locations in Germany (Ackerboden WF, Wald boden, Einheitserde, Novoment, Li35b, Heide Ackerboden, Magdeburg and Herrenhausen). Agar plates containing emulsified polymers (PCL or BTA 45:55 (Ecoflex) were used for counting the polymer degrading microorganisms (see 7.2.3.1) in eluates from the soils.

Preparation of the soil suspension

10 g soil were weighted in 250 ml bottles and were mixed with 90 ml MSV liquid medium (at RT) . The bottles were closed was turned 5 times over head. After that, the soil suspension was shaked at 100 rpm and room temperature for 20 minutes. The suspension was turned over head 5 times and was passed through a sieve (pore: 1,7 mm). The suspension was left for 5 min at room temperature for sedimentation. After 5 minutes, the supernatant was collected with a pipette. After that, the dilution series was prepared in glass tubes containing 9 ml of MSV medium (from $1:10^1$ to $1:10^3$).

c. Culture on plates

A 100 µl aliquot of each diluted sample was spreaded on MSV-agar medium plate containing emulsified polymers (PCL or BTA 45:55 (Ecoflex) with 0.1% w/v) and on complex medium (standard count agar) for counting all microorganisms in the soil samples (CFU).The cultures were incubated at 30°C for 30 days. PDMs (polymer degradation microorganisms) were identified an counted as colonies exhibiting a clear zone around the colony. On the other hand, on the standard count agar the colonies were also counted. After that, the percentage of the biodegradable microorganisms for PCL and BTA 45:55 (Ecoflex) were calculated by dividing the total number of clear zone by the number of total culturable colonies on standard count agar medium. The experiments were repeated three times.

7.19. Chemicals and apparatuses

The source of chemicals and the different apparatuses are listed in Table 7.9 and 7.10, respectively.

Table 7.9. List of chemicals used throught this work

Chemicals	Source
Salts, acids, alkalis, etc.	Merck, Darmstadt, Germany,
With a purity degree of > 95%	Riedel de Häen, Seelze, Germany,
	Fluka, deisenhofen, Germay,
	Sigma, Deisenhofen, Germany
Bis-acrylamide Solution	Roth, Karlsruhe, Germany
Bovine serum albumin	Sigma, Deisenhofen, Germany
Cultivation media:	Merck, darmstadt, Germany
	Difco, Hamburg, Germany
SDS-PAGE standard	BioRad, München , Germany.
Polyester	see table 6.1

Table 7.10. List of apparatuses used throughout this work

Apparatus	source
Incubator (model 500)	Memmert, Schwachbach, Germany
Cool- incubator	Memmert, Schwachbach, Germany
Shaking incubator	Adolf Kühner AG, Birsfelden, Germany
Millipore-Q-unit	Millipore, Eschborn, Germany
Thermomixer 5436	Eppendorf, Hamburg, Germany
Vacuum incubator	VT 5042Heraeus Holding GmbH, Hannover, Germany
Titration unit	Radiometer, Kopenhagen, Danemark
	pHM290 standard pH-Meter; Radiometer, Kopenhagen, Danemark
	Autbürette ABU901; Radiometer, Kopenhagen, Danemark
	pH-elektrod, Inlab 423, Mettler, Urdorf/ZH, Schweiz
	Thermostat M3, mgm Lauda, Lauda-Königshofen
	Becker Computer; 286er, 16MHz
Ultraturrax	TP 25, Jahnke & Kunkel KG, IKA-Werk, Staufen i. Breisgau
Centrifuges:	
	Eppendorf-Centrifuge 5417, Eppendorf, Hamburg, Germany
	Superfuge 22, Heraus Holding GmbH, Hannover, Germany
	Biofuge 17 Rs, Germany
Spectrophotometer	Perkin-Elmer lambda 15, Überlingen, Germany
	Ultrospec 300 pro, amersham Pharmacia biotech, Germany
Vortex	Vortex Genie 2^{TM}, Bender & Hobein AG, Zürich, Schweiz.

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9. Appendix

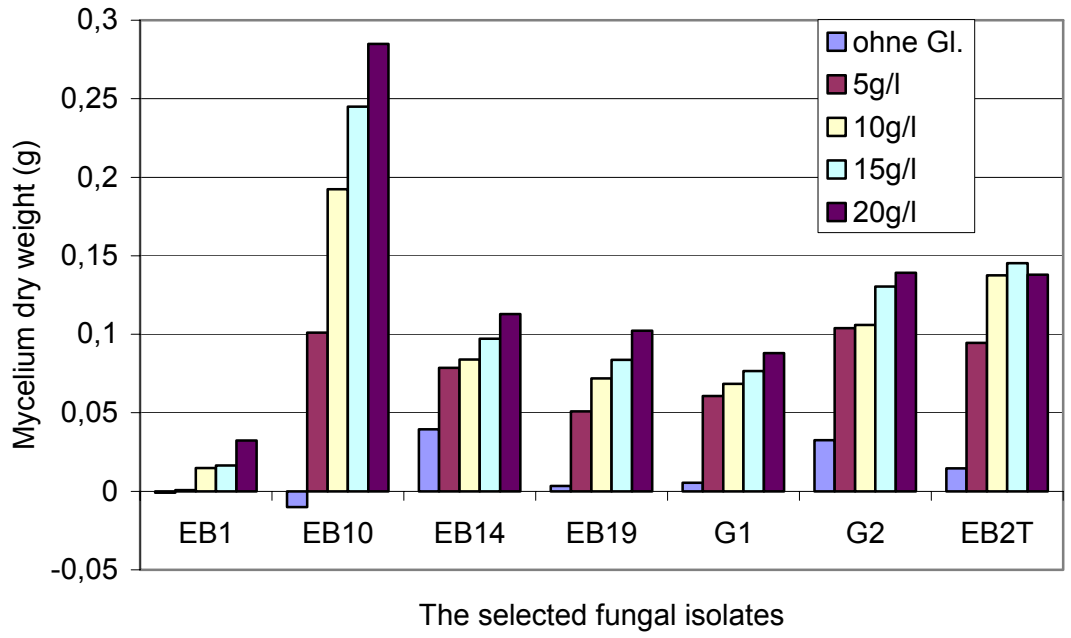

Fig. 9.1. Effect of different concentration from glucose on mycelium dry weight (g) for the selected fungal isolates
Medium: 30ml mineral salt medium (MSV)/flask (100ml), Temperature: at 30°C for EB1, EB10, EB14, EB19, G1, G2 but at 15°C for EB2T., Inoculum: 1 disc (5mm) from complete medium bearing the fungal growth, Incubation time: 1 week

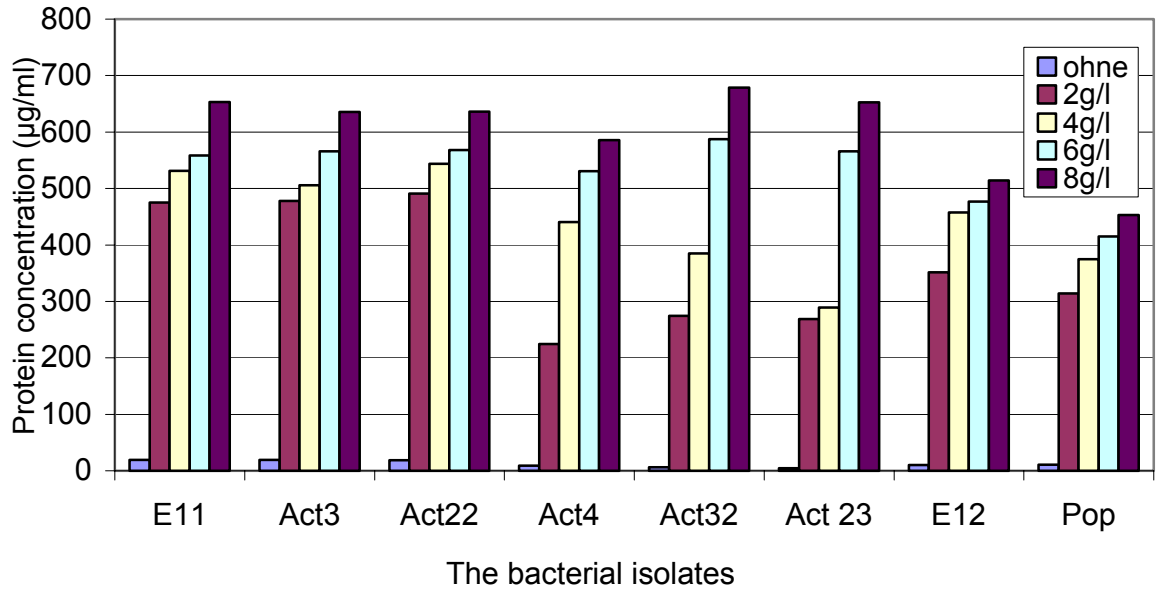

Fig.9.2. Effect of different concentration from glucose on the growth for the selected bacterial isolates
Medium: 30ml mineral salt medium (MSV)/flask (100ml), Temperature: 30°C with shaking at 160 rpm, Inoculum: 1 ml (10^5- 10^7 CFU/ml)from complete (GYM or LB), Incubation time: 3- 4 days

9.1. Distribution of BTA45:55 (Ecoflex) and PCL degrading microorganisms in different soils by using clear zone formation technique at 30°C

The distribution of BTA45:55 (Ecoflex) degrading microorganisms in different soil samples was compared with that of PCL-degrading microorganisms. The soil samples were collected from different locations in Germany. The depolymerizing potential was expressed by clear zone formation with the polyesters emulsified in MSV-agar. Results of the number of polymer degrading microorganisms (PDMs) in the 8 different soils are summarized in Fig.9.3. PDMs for PCL and BTA45:55 (Ecoflex) at 30°C were detected in almost all samples. Especially PCL degraders were found in all samples, and accounted for more than one tenth of the heterotrophic population in most cases (determined as total colony forming units). On the other hand, the number of BTA45:55 (Ecoflex) degraders were one hundredth of the heterotrophic population. This suggest that the potential to be degraded in the natural environment is significantly lower for a copolyester compared to a plastic composed of aliphatic polyesters. Also Nishida et al., (1998) and Ishigaki et al., (2000) demonstrated that PCL degrading microorganisms can be found in various environments.

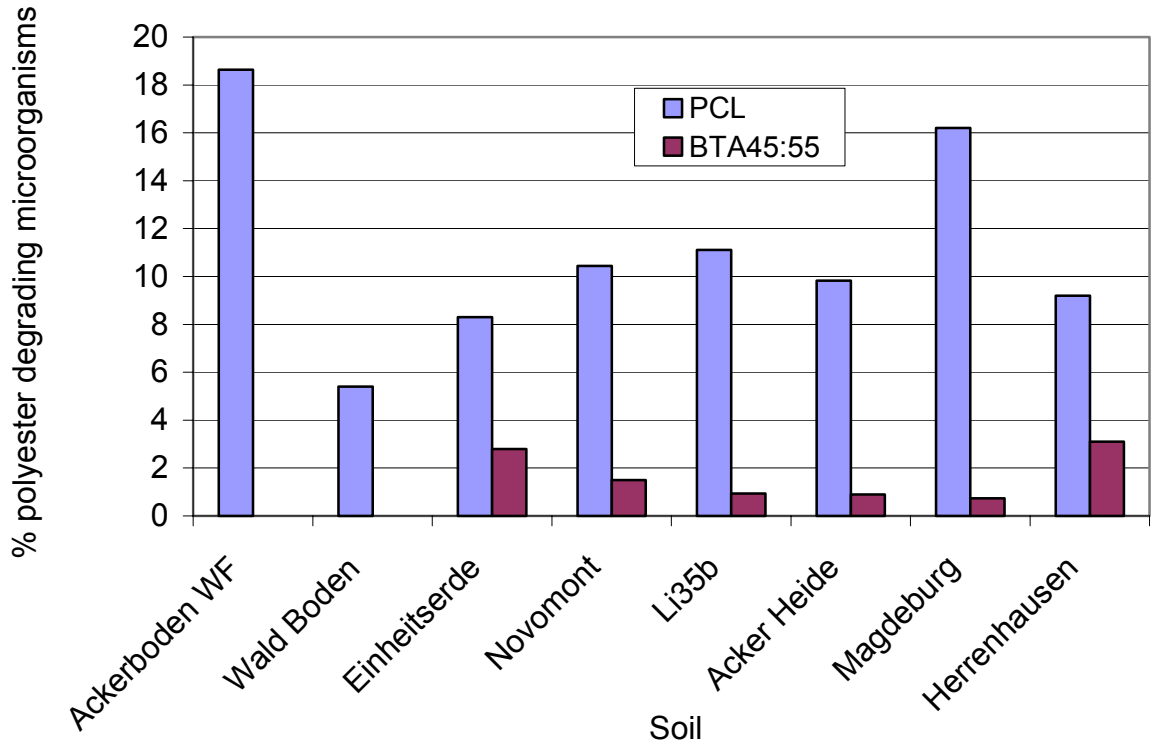

Fig.9.3. The % of the biodegradable microorganisms for Ecoflex- and PCL- from different soils determined by clear zone formation in mineral salt agar medium supplemented with the polyesters

(the percentage of the biodegradable microorganisms for PCL and BTA45:55 (Ecoflex) were estimated by account the total number of clear zone on emulsified MSV- agar medium with the polyesters to the number of total culturable colonies on standard count agar medium)

Medium: Mineral salt vitamin agar with BTA45:55 (Ecoflex) or PCL (0.1 % w/ v), Temperature: 30°C, Incubation time: 30 days, Inoculum: 100 µl of soil extract / plate